STUDIES ON ISOCITRATE LYASE GENE PROMOTER OF *CANDIDA TROPICALIS* : ITS REGULATION AND APPLICATION TO HETEROLOGOUS GENE EXPRESSION

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PREFACE

This is a thesis submitted by the author to Kyoto University for the degree of Doctor of Engineering. The studies collected here have been carried out under the direction of Professor Atsuo Tanaka in the Laboratory of Industrial Biochemistry, Department of Industrial Chemistry and in the Laboratory of Applied Biological Chemistry, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, during 1992–1999.

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CONTENTS

INTRODUCTION

Yeast is a beneficial microorganism for mankind because yeast has been used, for thousands of years, to make our daily foods such as bread, wine or beer, and at present a wide variety of biochemicals are also produced. Yeast is a eukaryotic microorganism which grows rapidly in simple and inexpensive media, and approximately 700 yeast species are presently recognized. Since gene manipulation technique of yeast is well-developed, certain kinds of yeast strains can be handled like *Escherichia coli*. Moreover, the cellular processes of yeast (for example, cell cycle regulation, biogenesis of subcellular organelles, structure of cytoskeleton, and protein secretion mechanism) are mechanistically highly conserved with other eukaryotic species including mammalian cells. Therefore, yeast is at one hand recognized as a useful tool for biotechnology, and on the other hand as a model organism for studying the basic problems in eukaryotic molecular biology.

In the present study, the author, focusing on a promoter region derived from an *n*-alkane-assimilating yeast *Candida tropicalis* (*UPR-ICL*), has investigated its application to heterologous gene expression in *Saccharomyces cerevisiae*, and further examined to make clear its regulation mechanism by utilizing two yeast strains, *Candida tropicalis* and *Saccharomyces cerevisiae*.

1. Heterologous gene expression in yeast

As a eukaryotic microorganism, yeast shares many common cellular mechanisms with higher eukaryotes and, therefore, it enables native conformation of eukaryotic exogenous proteins or has potential of executing posttranslational modification or secretion of proteins (Table 1). Yeast can grow quickly to a high cell density at lower cost than any other eukaryotes, and its genetic manipulation is simple and well-developed (Table 1). Because of these characteristics, yeast attracts many biotechnologists for its use as a host of foreign protein production. The yeast strain mostly used for this purpose is *Saccharomyces cerevisiae* which is called as bakers' or brewers' yeast. As for *Saccharomyces cerevisiae*, ample genetic techniques and information accumulated are available through a long history of its use [1, 2]. Moreover,

Table 1. Comparison of the features of commonly used expression systems.
**Escherichia coli*; ***Saccharomyces cerevisiae*; ++, Excellent; +, Good; -, Not good

Feature	Gene expression system			
	*E.coli**	Insect cells	Mammalian cells	*S.cerevisiae***
Growth in cheap media	++	-	-	++
Growth rate	++	-	-	++
Gene manipulation	++	-	-	++
Posttranslational modification	-	+	++	+
Secretion	-	++	++	++
Safety against human	-	+	+	++

since *Saccharomyces cerevisiae*, which is known as an organism having a status of generally regarded as safe (GRAS), has no pathogenicity by itself nor contains any detectable endotoxins or virus against human, it is a suitable host for the production of pharmaceutical or food products (Table 1) [1, 2].

For optimal gene expression, the right choice of transcriptional promoter is particularly important on the quantity of foreign protein production in the cells [1, 2]. Presently in *Saccharomyces cerevisiae*, powerful promoters are generally derived from the genes encoding glycolytic enzymes, e.g. phosphoglycerate kinase (*PGK*) [3], glyceraldehyde-3-phosphate dehydrogenase (*GAP*) [4], and alcohol dehydrogenase (*ADH1*) [5]. *PGK* mRNA, for example, is known to accumulate to 5% of all mRNA [6]. Although these promoters are slightly induced by glucose, they are significantly transcribed in other culture conditions as well. As a result, they are called as "constitutive" promoters (Table 2).

As foreign proteins are frequently harmful to the cell growth, a "regulative" promoter rather than constitutive one is desirable for heterologous gene expression in order to separate protein production from cell growth [1]. Promoters from the genes of galactose-metabolizing enzymes, e.g. galactokinase (*GAL1*) [7] or galactose transferase (*GAL7*) [8], are widely employed as powerful and regulative promoters in *Saccharomyces cerevisiae*. Transcription from these promoters are rapidly induced more than 1000-fold on the addition of galactose (Table 2) [9]. For the induction, binding of Gal4 protein, a transcriptional activator encoded by *GAL4*, to upstream activation sequence (UAS) on *GAL* promoter is necessary, and this

Table 2. **Relative strength of promoters functional in *Saccharomyces cerevisiae* on different carbon sources.** -, not induced; +, induced (the number of '+' indicates the strength of induction); nr, not reported; nt, not tested

Carbon source	Constitutive promoter	Regulative promoter	
	PGK, GAP	*GAL*	*UPR-ICL*
Glucose	++++	-	-
Galactose	nr	+++	nt
Glycerol	++	+	+
Acetate, Ethanol	++	+	+++

binding is controlled by galactose [10]. Several regulative promoters besides *GAL* promoters are present in *Saccharomyces cerevisiae*, such as the *PHO5* promoter which is induced 200-fold in the absence of phosphate [11] or the *CUP1* promoter which is induced 20-fold in the presence of Cu^{2+} [12], although these promoters are not so strong as *GAL* promoters. From the increasing demands on the different kinds of foreign protein production in yeast, it is significant to find new regulative promoters which are as strong as, or even stronger than *GAL* promoters, and having different regulation mechanisms.

2. Peroxisome and *n*-alkane-assimilation in *Candida tropicalis*

Candida tropicalis is an asporogenic diploid yeast which is characterized by its ability to utilize *n*-alkanes as a sole carbon and energy source. In assimilating *n*-alkanes, *Candida tropicalis* shows a change in its intracellular structure by proliferating a large number of peroxisomes (Fig. 1) [13, 14]. Peroxisome is a versatile, single-membrane-bound organelle occurring in most of eukaryotic cells. In *Candida tropicalis*, incorporated *n*-alkanes are first oxidized to alcohols by cytochrome P450 localized at endoplasmic reticulum, and the resulting alcohols are then transported into peroxisomes where oxidation into fatty acids *via* aldehydes occurs. The fatty acids are then activated to CoA esters and degraded by the β-oxidation system in peroxisome to produce acetyl-units. Since the development of peroxisome can be controlled easily by changing carbon sources in the medium, *Candida tropicalis* has been used as a model organism for investigating the function and biogenesis of peroxisome [15].

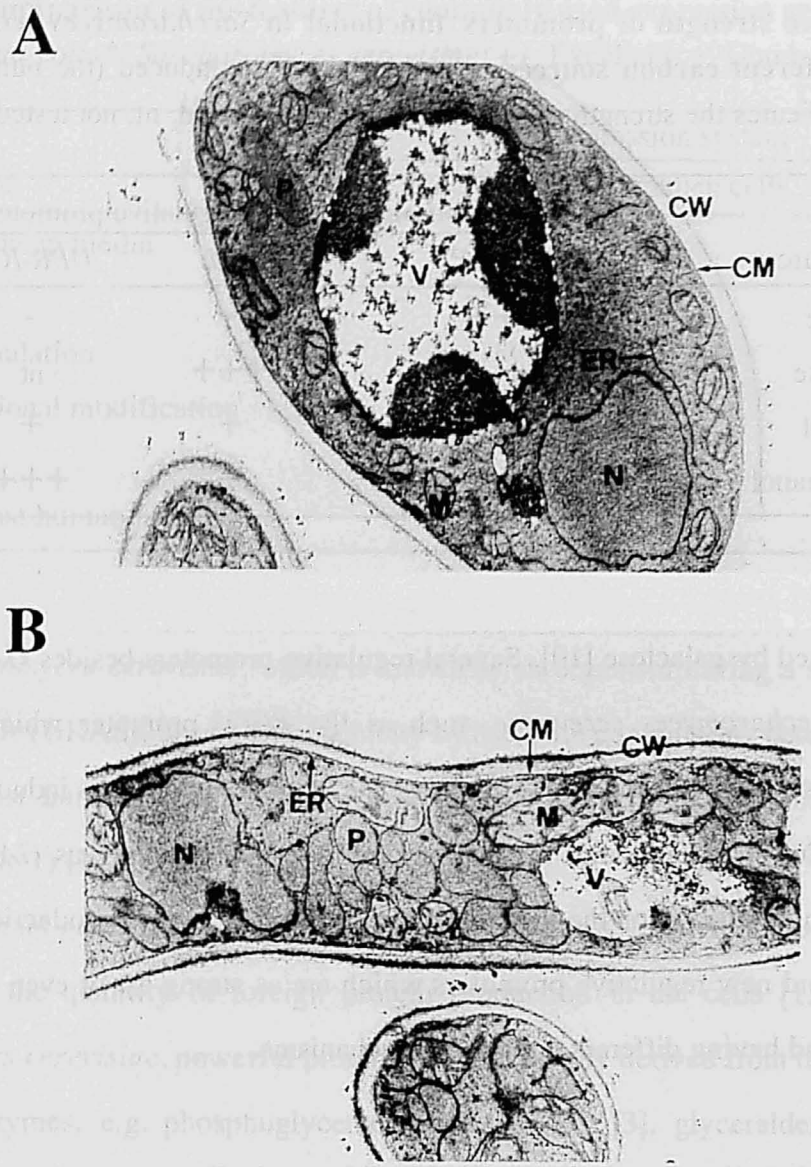

Fig. 1. Electronmicrographs of glucose- (A) and *n*-alkane- (B) grown cells of *Candida tropicalis* pK233. CM, cell membrane; CW, cell wall; ER, endoplasmic reticulum; M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole.

3. Regulation of peroxisomal proteins in *Candida tropicalis*

Peroxisome of *Candida tropicalis* contains enzymes necessary for degrading fatty acids, such as catalase, enzymes of the β-oxidation system, carnitine acetyltransferase, and key enzymes of the glyoxylate pathway (Table 3) [15]. Concomitant with peroxisome proliferation, the activities of these enzymes are induced in *n*-alkane-grown cells. Some of these enzymes are also induced during the cultivation on butyrate or propionate, both of which can induce peroxisome proliferation [16-19]. On the other hand, they are synthesized at low rate or not at

Table 3. Levels of peroxisomal enzyme activities of *Candida tropicalis* pK233.
N.D. ; not detected.

	Enzyme Activity (nmol min^{-1} mg^{-1})		
	(A) Glucose	(B) *n*-Alkane	(B) / (A)
Catalase	$1.7x10^5$	$4.7x10^6$	27
Long-chain alcohol dehydrogenase	6	80	13
Long-chain aldehyde dehydrogenase	9	98	11
Acyl-CoA synthetase	37	70	1.9
(β-Oxidation enzymes)			
Acyl-CoA oxidase	6.2	2500	400
Enoyl-CoA hydratase	55	5700	100
3-Hydroxyacyl-CoA dehydrogenase	N.D.	240	-
3-Ketoacyl-CoA thiolase	67	190	2.8
(Glyoxylate cycle enzymes)			
Isocitrate lyase	170	530	3.1
Malate synthase	23	65	2.8
NAD-linked glycerol-3-phosphate dehydrogenase	11	170	15
Carnitine acetyltransferase	530	9900	19
Uricase	42	180	4.3
D-Amino acid oxidase	110	73	0.7

all in glucose-grown cells (Table 3). This phenomenon is called as glucose repression (or catabolite repression) and is a ubiquitous event both in prokaryotic and eukaryotic microorganisms. Glucose represses the synthesis of a large number of enzymes that are required for the utilization of alternative carbon sources. This response is thought to be an energy-saving process and allows microorganisms to cope effectively with changes in carbon sources present in their environment.

Isocitrate lyase (ICL), a key enzyme of the glyoxylate cycle being localized in peroxisome of *Candida tropicalis*, is under the control of glucose repression (Table 3). ICL activity of *Candida tropicalis* is especially higher in the cells grown on acetate, and higher in the cells grown on *n*-alkane than in glucose-grown cells [16, 20]. Immunochemical titration shows that the change of ICL activity is not through inactivating the enzyme, but through adjusting the extent of the enzyme [16]. Northern blot analysis revealed that the ICL mRNA is rich in the cells grown on acetate and *n*-alkane but poor in glucose-grown cells [21]. These results suggest that there exists a genetic mechanism underlying glucose repression in *Candida tropicalis* which regulates the ICL gene in response to carbon sources. However, the

phenomenon of glucose repression requires strict control of multiple processes, including sensing of glucose, signal transduction or regulation of target genes, although each mechanism is not fully clarified.

4. Genetic mechanism underlying glucose repression of *Saccharomyces cerevisiae*

Because of its developed genetics and physiological background, the mechanism of yeast glucose repression has been studied extensively in *Saccharomyces cerevisiae* [22-27]. Recently in 1996, the whole genome of *Saccharomyces cerevisiae* was sequenced as the first completed eukaryotic genome [28, 29], which further accelerates the use of this organism as a model of eukaryotic cells.

Promoters of *Saccharomyces cerevisiae* controlled by the glucose repression mechanism were used to search for *cis*-regulatory elements responsible for derepression of gene expression. In the case of *ICL1* encoding an isocitrate lyase of *Saccharomyces cerevisiae*, a positive element called carbon source-responsive element (CSRE) was determined (Fig. 2) [30] and its consensus sequence was suggested as CCRTYCRTCCG (where R = A or G, Y = C or T) [31, 32]. Similar sequence motifs were also identified in the upstream regions of *FBP1* encoding fructose 1,6-bisphosphatase [33-35], *PCK1* encoding phospho*enol*pyruvate carboxykinase [36], *MLS1* encoding malate synthase [31], and *ACS1* encoding acetyl-coenzyme A synthetase [32]. However, protein factor(s) which specifically binds to the CSRE is not identified to date.

Various *trans*-acting factors which are involved in the genetic regulation of the glucose repression in *Saccharomyces cerevisiae*, were also identified (Fig.2). *SNF1* (Sucrose Non-Fermenting) was first identified by a screening for *Saccharomyces cerevisiae* mutants unable to grow on sucrose as the sole carbon source [37, 38]. Other than sucrose, *snf1* mutant of *Saccharomyces cerevisiae* does not grow on galactose, raffinose or nonfermentable carbon sources such as glycerol, ethanol and acetate [37]. In the *snf1* mutant cells, derepression of the genes encoding enzymes of alternative sugar utilization (*GAL* or *SUC* genes), gluconeogenesis (*FBP1* and *PCK1*) or glyoxylate cycle (*ICL1* and *MLS1*) is defective [37, 39, 40]. Snf1 protein is a serine/threonine protein kinase interacting with bridge protein (Gal83, Sip1 or Sip2 proteins) and Snf4 protein to constitute a protein complex [41, 42]. This kinase activity of the complex varies by the glucose concentration in the medium; it is

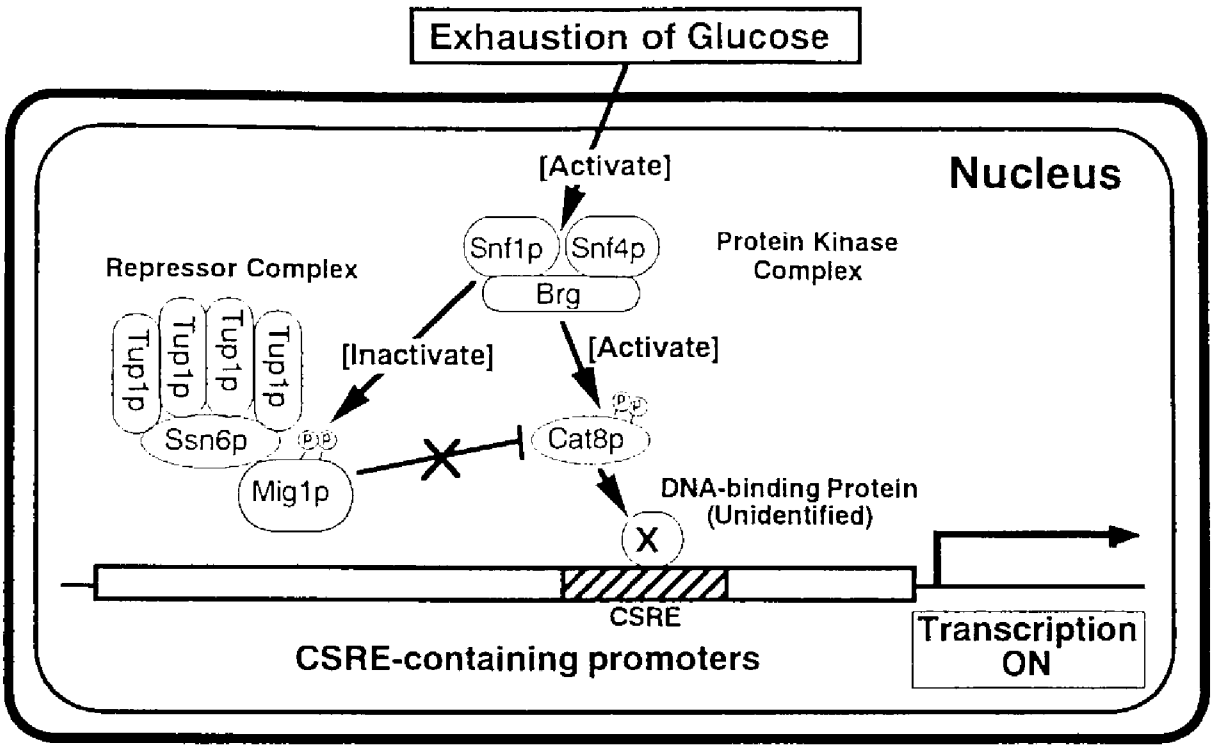

Fig. 2. Transcriptional factors involved in the regulation of CSRE-containing promoters of *Saccharomyces cerevisiae*. Brg, bridge protein (Gal83, Sip1 or Sip2 protein); Ⓟ, indication of phosphorylation; CSRE, carbon source-responsive element

activated in the absence of glucose and inactivated in the presence of it [43], and this kinase activity is absolutely necessary for the derepression [44]. In addition to *SNF1*, *CAT8* (CATabolite repression) was identified as a gene necessary for the derepression of *ICL1*, *FBP1* or *PCK1*, but not for that of *GAL* or *SUC* genes [45, 46], and is under the control of *SNF1* [45, 46]. Recent evidence shows that the derepression of *FBP1* and *PCK1* has a correlation with the phosphorylation of Cat8 protein [47]. Although this phosphorylation is dependent on *SNF1* [47], whether or not the Snf1 protein kinase complex directly phosphorylate Cat8 protein is not clear. As for the factors concerning the repression on glucose, *MIG1* (Multicopy Inhibitor of *GAL1* promoter) encoding a zinc-finger DNA-binding protein was identified as necessary for the repression of *SUC2* or *GAL* genes in glucose-grown cells [48, 49]. Mig1 protein constitutes a repressor complex with one molecule of Ssn6 protein and four molecules of Tup1 protein [50, 51]. In the absence of glucose, Mig1 protein is inactivated by the Snf1 protein-dependent phosphorylation [52]. Mig1 protein is also responsible for the repression of *CAT8* in glucose-grown cells [47, 53], while no Mig1 protein-binding sites are identified on the *ICL1*, *FBP1* or *PCK1*. Therefore, Mig1 protein partly contributes to the derepression

of CSRE-regulated gene expression by way of the derepression of *CAT8*.

5. Regulation of *UPR-ICL* in *Saccharomyces cerevisiae*

When the ICL gene of *Candida tropicalis* was introduced into *Saccharomyces cerevisiae* together with its 5'-upstream region (*UPR-ICL*) and the cells were cultivated on glucose and non-fermentable carbon sources, recombinant ICL protein appeared in the cells grown on non-fermentable carbon sources, while the cells grown on glucose produced little recombinant protein [54-57]. Especially in the cells grown on acetate, recombinant ICL protein occupied as much as 36% of the total soluble protein in the cells [54]. These results suggest a potential ability of *UPR-ICL* being applied for heterologous gene expression in *Saccharomyces cerevisiae* as a powerful and tightly-regulated promoter.

Moreover, the fact that *UPR-ICL*-mediated transcription is still glucose-repressive in *Saccharomyces cerevisiae* demonstrates that similar mechanisms are commonly existing in both of the yeast strains concerning the regulation of ICL gene. In order to determine the region of *UPR-ICL* which supports strong and regulative gene expression in *Saccharomyces cerevisiae*, various deletion fragments of *UPR-ICL* were constructed [55, 57]. Two sequence regions were determined which can independently activate gene expression in acetate-grown cells (region A1 and region A2). Within region A2 (-370 to -356), there exists a sequence motif similar to CSRE, whereas region A1 (-728 to -569) does not contain such motif [57]. These two regions are both responsible for the activation of *UPR-ICL*-mediated transcription in *Saccharomyces cerevisiae,* but their mechanisms appear to be different. Activation through region A2 is dependent on both *SNF1* and *CAT8*, whereas activation through region A1 is only *SNF1*-dependent [57]. At present, factors other than Snf1 protein and Cat8 protein, which are involved in the regulation of *UPR-ICL*-mediated transcriptional activation in *Saccharomyces cerevisiae*, are not found.

In the present study, the author has inspected the possibility of *UPR-ICL* to be applicable to heterologous gene expression in *Saccharomyces cerevisiae*. The author considers the availability of *UPR-ICL* as a regulative promoter used for foreign protein production in *Saccharomyces cerevisiae*, and actually some of the foreign proteins from bacteria and mammals are overexpressed using this system both in intracellular and extracellular spaces. Furthermore,

the author has also studied the fundamentals of regulation mechanisms of *UPR-ICL* in response to carbon sources. A novel factor was found in *Saccharomyces cerevisiae* and the mechanism it concerns on regulating *UPR-ICL* has been investigated. Moreover, an *SNF1* homolog was cloned from *Candida tropicalis* and its function on the regulation of *UPR-ICL* has been studied in *Candida tropicalis*.

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SYNOPSIS

Part I. Application of the upstream region of isocitrate lyase gene of *Candida tropicalis* (*UPR-ICL*) to heterologous gene expression in *Saccharomyces cerevisiae*

When isocitrate lyase (ICL) gene of an *n*-alkane-assimilating yeast, *Candida tropicalis*, is introduced into *Saccharomyces cerevisiae* together with its 5'-upstream region (*UPR-ICL*), a large amount of recombinant ICL protein was produced in the cells grown on non-fermentable carbon source such as acetate, ethanol, while little was produced in the cells grown on glucose. This indicates that *UPR-ICL* could function as a regulative promoter in *Saccharomyces cerevisiae* in response to non-fermentable carbon sources.

Chapter 1 deals with the expression of two foreign genes coding for β-galactosidase from *Escherichia coli* (*LacZ*) and the smaller isoform of glutamate decarboxylase from rat brain (*GAD65*) under the control of *UPR-ICL*. The vector expressing *LacZ* under the control of *UPR-ICL* was constructed and introduced into *Saccharomyces cerevisiae*. The β-galactosidase activity in the cell lysate was repressed by glucose and enhanced over 300-fold by acetate, and the amount of recombinant protein was comparable to that produced using *GAL1* promoter. An expression vector, pWI3, was constructed which contains multicloning site between *UPR-ICL* and the transcriptional terminator of the isocitrate lyase gene (*TERM-ICL*). Using pWI3, glutamate decarboxylase was highly produced as a soluble and active form. These results demonstrate that the novel expression system using *UPR-ICL* and *TERM-ICL* is useful for the production of heterologous proteins in *Saccharomyces cerevisiae*.

Chapter 2 deals with the expression of human histidine decarboxylase (HDC) cDNA under the control of *UPR-ICL*. The vector for expressing HDC was constructed using pWI3, and introduced into *Saccharomyces cerevisiae*. HDC activity in the cell lysate was measured throughout the incubation period where the maximum HDC activity was obtained near the end of the log-phase. A typical preparation gave the specific activity of 210 (pmol min^{-1} mg^{-1}) in the crude cell lysate. The enriched activity of HDC in the soluble fraction suggests that yeast expression system provides the properly folded and catalytically active enzyme. Therefore,

the present system is suitable for the large scale production of the enzyme.

Chapter 3 deals with the expression and secretion of the cycloinulo-oligosaccharide fructanotransferase (CFTase) gene of *Bacillus circulans* MCI-2554 under the control of *UPR-ICL*. CFTase gene was fused to the secretion sequence of the α-factor and expressed in *Saccharomyces cerevisiae*. Secreted recombinant CFTase protein (*Sc*CFTase) was purified and characterized. *Sc*CFTase 2, the major product of the expression system employed, was shown to be N-glycosylated. *Sc*CFTase 2 showed an optimum pH, an optimum temperature, and a pH stability similar to those of CFTase purified from *Bacillus circulans* MCI-2554 (*Bc*CFTase), but exhibited a significant increase in thermostability. Over 50% of *Sc*CFTase 2 activity was retained even after 30 min incubation at 80 °C. This high thermostability was due to N-glycosylation of the protein. Next, a *Saccharomyces cerevisiae* strain was constructed which had two copies of CFTase gene integrated into its chromosomes (CF/HW2A). Production of *Sc*CFTase by the CF/HW2A strain reached 391 U per liter of culture at 120 h, which corresponded to 8.40 mg of protein per liter, by shake-flask cultivation.

Part II. Analysis of the transcriptional regulation mechanism of *UPR-ICL*

The fact that *UPR-ICL* is also functional in *Saccharomyces cerevisiae* demonstrates that similar mechanisms are commonly existing in both of yeasts concerning the regulation of ICL gene. In Part II, the author analyzes the transcriptional regulation mechanism of *UPR-ICL*, especially focusing on the isolation of regulatory factors which control *UPR-ICL*-mediated transcription.

Chapter 1 deals with the isolation and characterization of a new regulatory factor which is responsible for the derepression of *UPR-ICL*-mediated gene expression in *Saccharomyces cerevisiae*. A mutant was isolated which failed to derepress *UPR-ICL*-mediated gene expression in acetate medium. This mutant was also defected in derepressing the isocitrate lyase gene of *Saccharomyces cerevisiae* (*ICL1*). The gene that complemented this mutation (*FIL1*) was isolated, and its deduced amino acid sequence showed similarity to ribosome recycling factors of prokaryotes. At its N-terminus of Fil1 protein, there existed a mitochondrial-

targeting sequence. The subcellular fractionation of the Δ*fil1* strain showed that protein constituents of the mitochondrial fraction differed from those of the wild-type strain, but resembled those of chloramphenicol-treated cells or ρ° cells, suggesting that Fil1 protein is necessary factor for protein synthesis in mitochondria. Furthermore, the cells treated with antimycin A, along with Δ*fil1* cells, showed deficiency in derepression of isocitrate lyase. Northern blot analysis showed that this can be ascribed to no increase in transcription of *ICL1* and *FBP1* encoding fructose 1,6-bisphosphatase. These results indicate the presence of a communication pathway between mitochondria and the nucleus which represses expression of genes encoding the key enzymes of the glyoxylate cycle and gluconeogenic pathway when there is a deficiency in the mitochondrial respiratory chain.

Chapter 2 deals with the isolation and characterization of *SNF1* from *Candida tropicalis* (*CtSNF1*). *SNF1* of *Saccharomyces cerevisiae* is essential gene for the derepression of glucose repression including *UPR-ICL*-mediated gene expression. Sequence analysis of *CtSNF1* revealed that it encodes a 619-amino-acid protein (CtSnf1p), and the deduced amino acid sequence showed a notably high similarity (86.0% identity) with Snf1p of *Candida albicans*, while the identity with Snf1p of *Saccharomyces cerevisiae* was 61.2%. *CtSNF1* can complement the Δ*snf1* mutant of *Saccharomyces cerevisiae*, indicating CtSnf1p is a functional homolog. The method for introducing exogenous DNA into *Candida tropicalis* was used to construct a *SNF1/snf1* heterozygote strain (KO-1). In KO-1 cells, the activities of catalase and acyl-CoA oxidase were decreased 47 to 80 % against wild-type cells, while the activity of isocitrate lyase was almost comparable. Disruption of the second allele of *CtSNF1* was unsuccessful, and therefore, a strain in which the promoter region of *CtSNF1* is replaced with *GAL10* promoter was constructed (KO-1G). KO-1G can normally grow on galactose, while growth retardation was observed for the other carbon sources including glucose. These results indicates that *CtSNF1* is an essential gene for the cell growth.

Part I Application of the upstream region of isocitrate lyase gene of *Candida tropicalis* (*UPR-ICL*) to heterologous gene expression in *Saccharomyces cerevisiae*

Chapter 1. Development of a novel heterologous gene expression system in *Saccharomyces cerevisiae* using *UPR-ICL*

INTRODUCTION

Yeast *Saccharomyces cerevisiae* is one of attractive hosts for producing heterologous proteins, because of its rapid growth in simple and inexpensive media, of its relatively easy gene manipulation like *Escherichia coli*, and of the extensive studies in genetics and physiology. Moreover, it is a eukaryotic microorganism which shares many common mechanisms with higher eukaryotes, including the posttranslational glycosylation of proteins etc. The choice of a transcriptional promoter being used has decisive effects on the quantity of foreign protein production in the cells [1]. At present, glycolytic promoters, e.g. *PGK* [2] and *GAP* [3] or galactose-regulated promoters, e.g. *GAL1* [4] and *GAL7* [5], are widely employed as powerful promoters for heterologous gene expression in *Saccharomyces cerevisiae*.

A genomic DNA fragment containing an 5'-upstream region of the isocitrate lyase (ICL) gene [*UPR-ICL*, 1530-bp], a coding region [1650-bp], and a termination region [*TERM-ICL*, 328-bp] from an *n*-alkane-assimilating yeast *Candida tropicalis*, was previously isolated [6]. When this DNA fragment was introduced into *Saccharomyces cerevisiae* using a plasmid with high copy number, and the cells were grown on acetate as an inducer of the enzyme synthesis [7], the ICL protein occupied as much as 36% of the total soluble protein in the cells [8]. Expression was also enhanced by other non-fermentable carbon sources, such as ethanol and glycerol/lactate, but repressed by glucose. Thus, *UPR-ICL* is functional in *Saccharomyces cerevisiae* [8]. Since an inexpensive compound, like acetate, can be an inducer and carbon source, the application of *UPR-ICL* would be attractive for foreign gene expression in *Saccharomyces cerevisiae*.

In this chapter, the author reports the construction of expression plasmids using *UPR-ICL* and the expressions of heterologous genes from *Escherichia coli* and mammalian tissue.

MATERIALS AND METHODS

Strains and plasmids

Escherichia coli DH5α [F⁻ *endA1 hsdR17*(r_K^-/m_K^-) *supE44 thi-1*, λ⁻ *recA1 gyr*A96 Δ*lacU169*(ϕ80*lac*ZΔM15)] was used as a host for recombinant DNA manipulation. *Saccharomyces cerevisiae* strain MT8-1 (*MATa ade his3 leu2 trp1 ura3*) [9] was used as a host for the protein production.

Construction of expression vectors

(1) Construction of the plasmid (pMIZ21) for the expression of *LacZ* (See Fig. 1)

The DNA fragment without ICL coding region was prepared by polymerase chain reaction (PCR) from B1-pUC19, a plasmid which contained 1530-bp *UPR-ICL*, coding region, and 328-bp *TERM-ICL* at the *Bam*HI site of pUC19. A primer, which hybridized at 35-bp downstream of the initiation codon, is 5'-GCTTCTTCTTGGTTGATGTCGATC-3'. A primer, 5'-TCCATGGAAACCAAGGCTAAGG-3', used to introduce *Nco*I site, hybridized at 24-bp upstream of the stop codon. The amplified fragment was digested with *Nco*I and self-ligated (pUI2). PCR was also used to introduce *Nco*I site at the both ends of *LacZ* gene, where pMC1871 (Pharmacia, Uppsala, Sweden) was used as the template. A primer, 5'-CCCATGGATCCCGTCGTTTTACAACG-3', which hybridized at the 5'-end of *LacZ* was designed to form an initiation codon. A primer, 5'-CGGATCCCCCATGGCCGGTTATTA-3', hybridized at the 3'-end. The PCR fragment was cut by *Nco*I and inserted into pUI2 (pUIZ21). The *Sal*I-*Sma*I fragment from pUIZ21 was inserted at the *Sal*I-*Pvu*II sites of high-copy shuttle vector pMT34(-G7) (pMIZ21). A pMT34(-G7) was prepared by removing *GAL7* promoter from pMT34(+3) [9].

(2) Construction of the plasmid (pGAD11) to express GAD65 cDNA (See Fig. 2)

An *Nco*I-*Eco*RI fragment from GAD65 cDNA of rat brain [10], whose initiation codon is at the *Nco*I site and *Eco*RI site locates about 400-bp downstream of the stop codon, was inserted at the *Nco*I-*Eco*RI sites of B1-pUC19 (pUIG11). pUIG11 was cut by *Eco*RI, treated with T4 DNA polymerase (blunting), then cut by *Sal*I. The resulting fragment containing *UPR-ICL*, GAD65 cDNA, and 3'-noncoding region of GAD65 cDNA, was inserted into the *Sal*I-*Pvu*II sites of high-copy shuttle vector pMT34(-G7) (pGAD11).

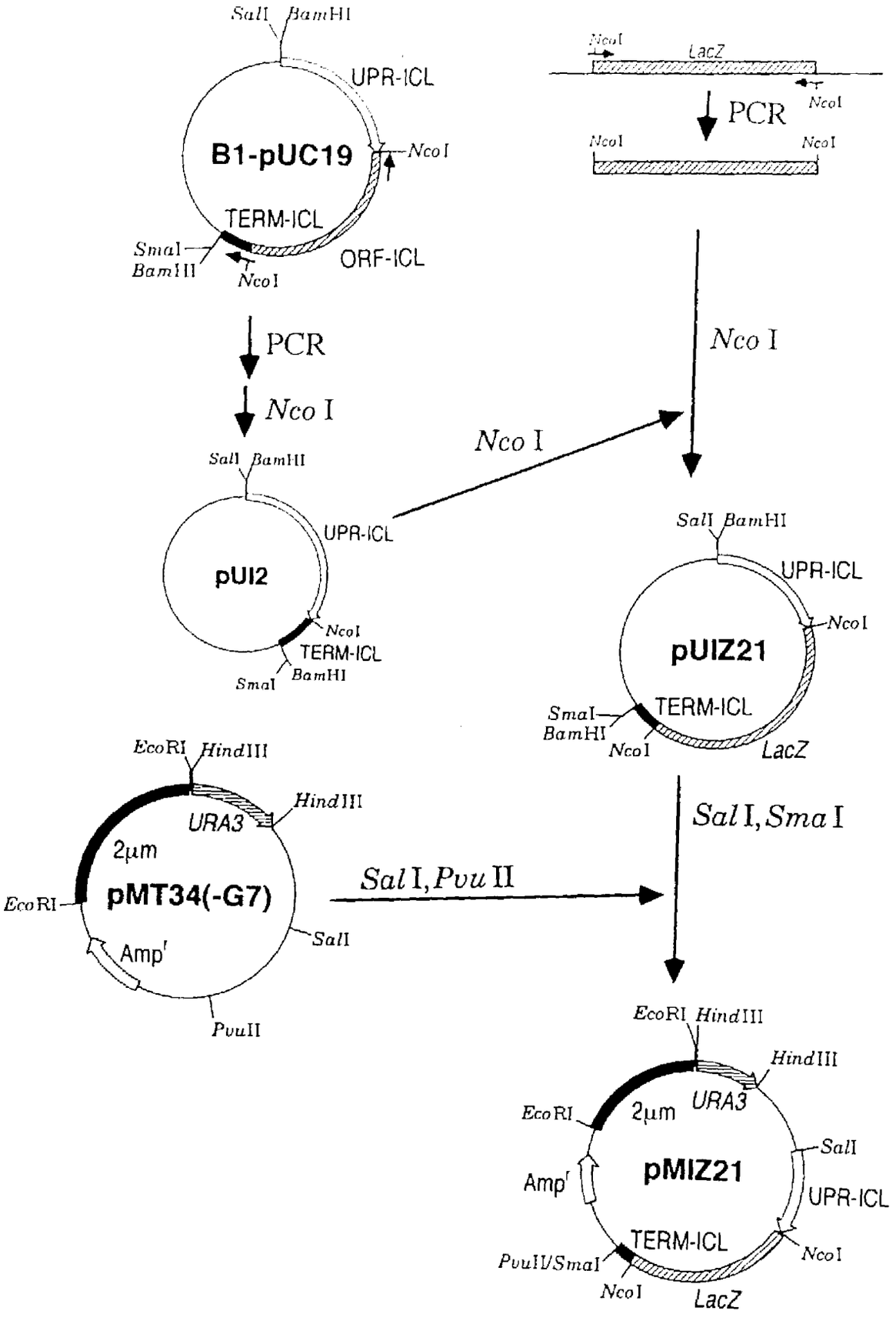

Fig. 1. Construction of the plasmid (pMIZ21) for the expression of *LacZ*. Primers used for PCR are indicated by arrows along the plasmid B1-pUC 19 and *LacZ*.

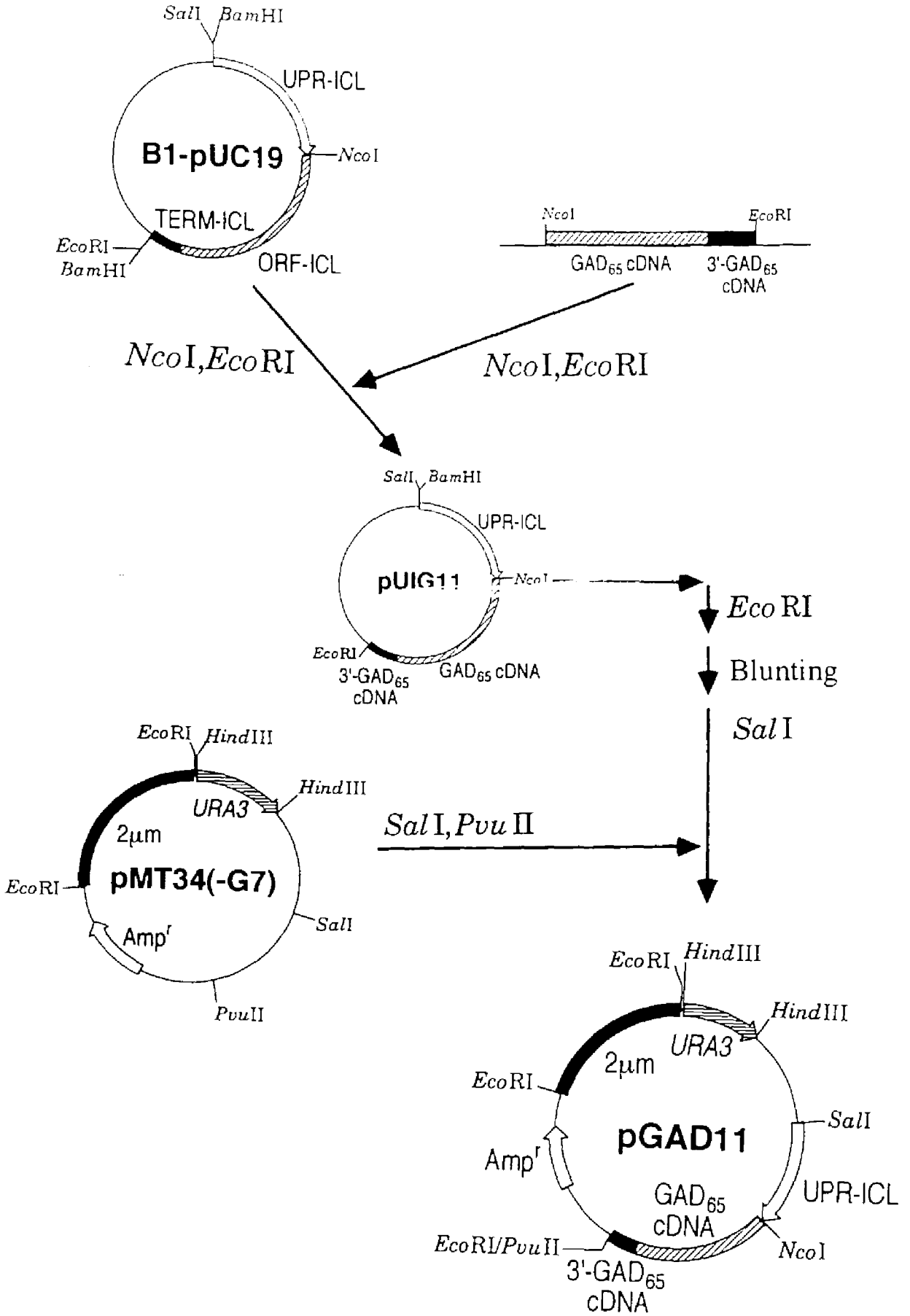

Fig. 2. Construction of the plasmid (pGAD11) to express GAD65 cDNA.

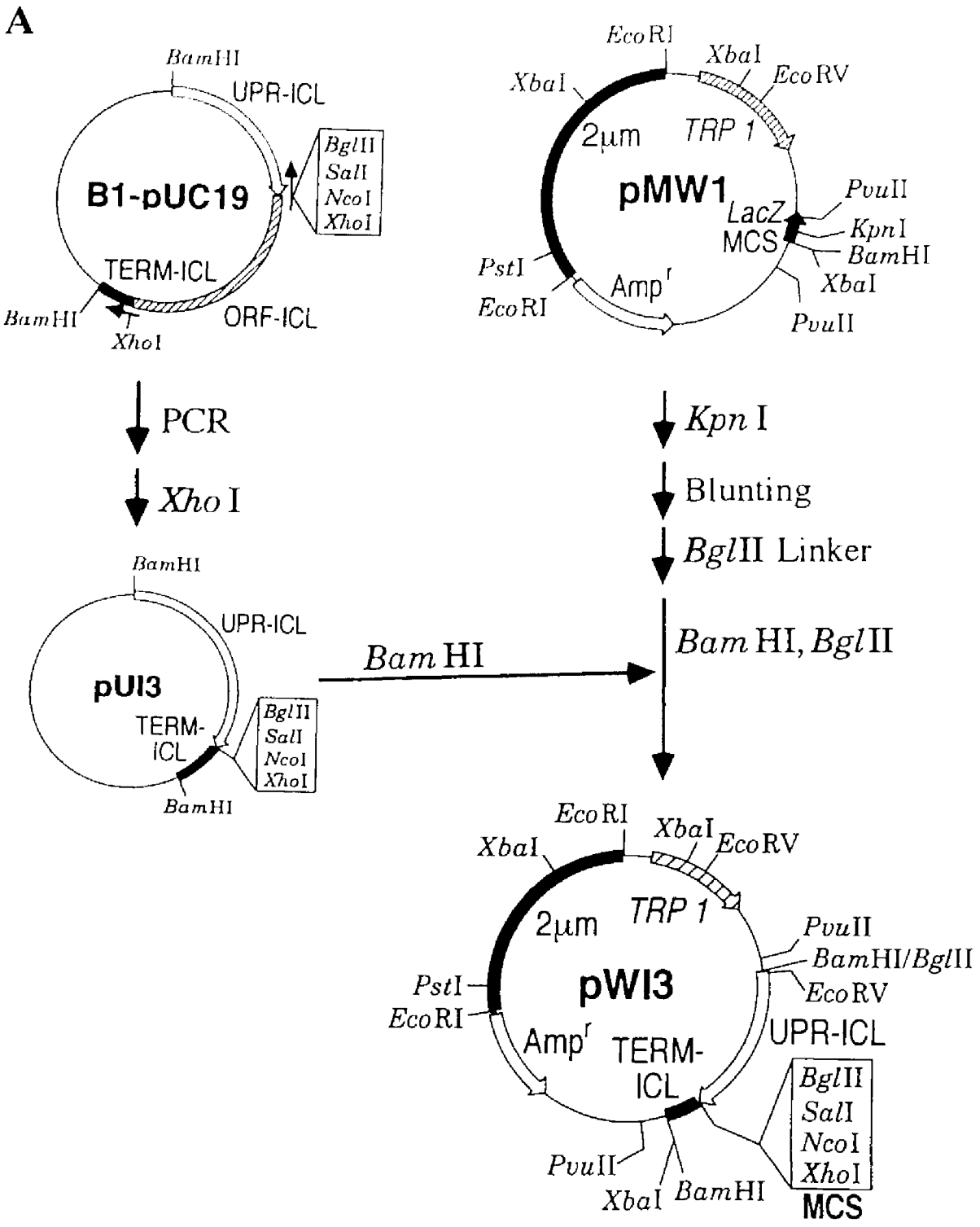

Fig. 3. Construction of the plasmid pWI3. In A, primers used for PCR and multicloning sites are indicated by arrows and MCS, respectively. In B, the nucleotide sequence around the junction of *UPR-ICL* and *TERM-ICL* is shown. The initiation codon, stop codon of ICL gene, and multi-cloning sites prepared are boxed. Primers are positioned at their annealing sites. Miss-match bases introduced in primers are underlined.

B

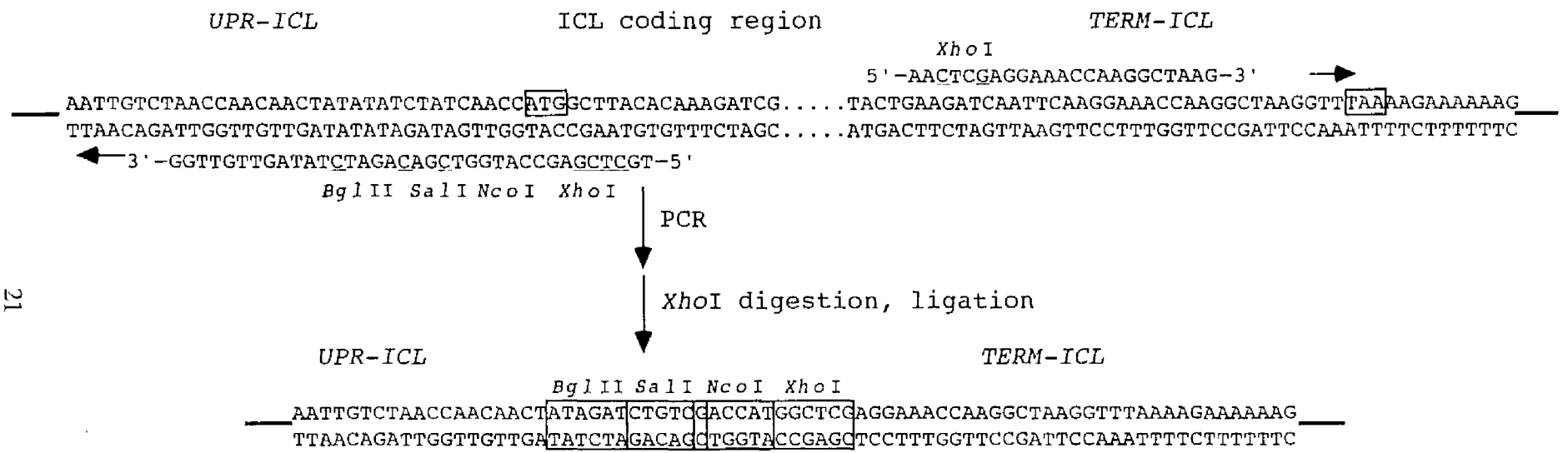

Fig. 3. (Continued).

(3) Construction of the plasmid (pWIG31) for the high-level expression of GAD65 cDNA

For the construction of the expression vector, pWI3 (See Fig. 3A), PCR was used to introduce multicloning sites between *UPR-ICL* and *TERM-ICL*. A primer, 5'-TGCTCGAGCCATGGTCGACAGATCTATAGTTGTTGG-3', which hybridized at the initiation codon, was used to introduce *Bgl*II, *Sal*I, *Nco*I, and *Xho*I sites. 5'-AACTCGAGGAAACCAAGGCTAAG-3', which hybridized at the stop codon, was used to introduce *Xho*I site. PCR fragment was digested with *Xho*I and self-ligated to make pUI3. *Bam*HI-*Bam*HI fragment from pUI3 containing *UPR-ICL*, the DNA fragment with multicloning sites and *TERM-ICL*, were introduced into high-copy plasmid pMW1 to make pWI3. pMW1 was constructed by introducing 2.14 kbp *Eco*RI-*Eco*RI fragment of pMT34(+3) containing a part of 2μm DNA into the *Aat*II site of yeast integrating vector pRS404 [11]. Furthermore, to construct the plasmid (pWIG31, Fig. 6A) for the high-level expression of GAD65 cDNA using pWI3, PCR was performed to introduce *Sal*I site at the downstream of the stop codon with pGAD11 as the template. A primer, 5'-CCTCCATTCCTCTTCTTGTC-3', hybridized inside of *UPR-ICL* (82-bp upstream of the initiation codon). A primer, 5'-TGGTCGACAAAGTGATTACAAATCTTGTCC-3', which hybridized at the stop codon, was used to introduce *Sal*I site. An amplified fragment was cut first by *Nco* I, where the initiation codon existed, and by *Sal*I, then inserted between the *Nco*I and *Xho*I sites of pWI3 (pWIG31).

Cultivation and preparation of cell-free extract

Yeast cells were precultivated in YPD medium [1% yeast extract (Difco, Detroit, MI, USA), 1% pepton (Difco) and 2% glucose], transferred to a minimal medium [0.67% yeast nitrogen base without amino acid (Difco) with appropriate supplements], containing 2% glucose (SD), 2.67% sodium acetate (SA) or 2% each of glycerol and sodium lactate (SGlyLac), and cultivated at 30 °C. Cells in the late-exponential phase were harvested and suspended in 50 mM potassium phosphate buffer (pH 7.2). This cell suspension was subjected to ultrasonic disintegration at 0 °C for 2.5 min, followed by centrifugation at 12,000 *g* for 20 min, and the resulting supernatant was used as cell-free extract.

Assay

β-galactosidase activity was measured basically. according to Craven et al. [12]. The

substrate solution was composed of 0.014 M *o*-nitrophenyl-β-D-galactopyranoside (ONPG) (Wako Pure Chemical Industry, Osaka, Japan) and 0.01 M $MgCl_2$ in 0.01 M Tris-acetate buffer (pH 7.5). This substrate solution (0.5 ml), 0.1 M potassium phosphate buffer (pH 7.0) (2.1 ml), and 1.0 M 2-mercaptoethanol (0.3 ml) were mixed well and incubated for 2.5 min at 30 °C. Reaction was started by the addition of 100 μl of the cell-free extract. Activity was determined from an increase in absorbance at 405 nm resulting from the hydrolysis of ONPG. Units (nmol/min) were calculated according to Miller [13].

The activity of glutamate decarboxylase was determined by measuring the formation of $^{14}CO_2$ from L-1-^{14}C glutamate essentially as described by Wu et al. [14]. In a typical assay, the incubation vessel (Kontes, Vineland, NJ, USA) contained 100 μl of the assay mixture consisting with 11.1kBq L-1-^{14}C glutamic acid (NEN, USA), 200 mM sodium glutamate, 1mM pyridoxal 5'-phosphate in 1M sodium phosphate buffer (pH 7.2). The plastic center well of the incubation vessel held 200μl of β-phenylethylamine (25 % in ethanol) to absorb CO_2 formed. The reaction was started by mixing 900μl of enzyme-containing solution to make a final volume of 1ml. Immediately after the enzyme addition, the reaction vessel was sealed with a rubber stopper and incubated for 30 min at 37 °C. The reaction was terminated by injecting 200μl of 0.5N H_2SO_4 into the reaction mixture. The vessels were incubated for another 60min to ensure a complete release of CO_2 and absorption in β-phenylethylamine. As a control, H_2SO_4 was added prior to the enzyme. ^{14}C counts obtained were converted to units (μmol/min) to represent the decarboxylase activity.

Electrophoresis and Western blot analysis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis were carried out by the method reported previously [15]. The molecular masses were estimated by using the following standard proteins : myosin, 212 kDa; γ_2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa; glutamate dehydrogenase, 53 kDa (Pharmacia), and the following prestained proteins: phosphorylase B, 106 kDa; bovine serum albumin, 80 kDa; ovalbumin, 49.5 kDa; carbonic anhydrase, 32.5 kDa; soybean trypsin inhibitor, 27.5 kDa; lysozyme, 18.5 kDa (BioRad, Hercules, CA, USA).

RNA blot hybridization analysis

RNA blot hybridization analysis was carried out as described Atomi *et al.* [6] using a biotin-labeled DNA probe. Detection was performed by luminescence reaction with Photo Gene Nucleic Acid Detection System kit [Bethesda Research Laboratories life Technologies (BRL), Gaithersburg, MD, USA]. Size markers used were as follows: 23 S and 16 S, ribosomal RNA from *Escherichia coli* ; 28 S and 18 S, ribosomal RNA from calf liver (Pharmacia).

RESULTS

Expression of β-galactosidase gene (*LacZ*) under the control of *UPR-ICL*.

In order to determine whether or not *UPR-ICL* could induce foreign gene expression, *LacZ* of *Escherichia coli* was inserted between *UPR-ICL* and *TERM-ICL* (pMIZ21) and expression of the gene was examined. Construction of pMIZ21 is shown in Fig. 1.

Saccharomyces cerevisiae cells transformed with pMIZ21 were grown in a minimal medium with either glucose (SD) or acetate (SA) as a sole carbon source. The cell-free extracts obtained by sonication were analyzed by SDS-PAGE. As shown in Fig. 4A, a band corresponding to a molecular mass of 116 kDa was observed by Coomassie brilliant blue staining only when the cells having pMIZ21 were grown on acetate. Western blot analysis by a polyclonal antibody against β-galactosidase confirmed that this band corresponded to therecombinant β-galactosidase (Fig. 4B).

The cells transformed with pMT34(-G7), a control plasmid which had no insert, showed no detectable β-galactosidase activity when grown in either SD or SA medium (Table 1). The cells having pMIZ21 exhibited a slight β-galactosidase activity when grown in SD medium, while the specific activity was enhanced over 300-fold when the cells were grown in SA medium. The expression level of β-galactosidase with *UPR-ICL* calculated on the basis of the purified β-galactosidase activity reached 6.5 % of the total soluble protein and was comparable to that of *GAL1* (5.5 % of the total soluble protein) [4], one of the most powerful and tightly-regulated promoters of *Saccharomyces cerevisiae*.

Expression of rat glutamate decarboxylase (GAD) cDNA under the control of *UPR-ICL*.

The expression of the smaller isoform of rat glutamate decarboxylase (GAD65) cDNA was carried out using *UPR-ICL* (A cDNA was a gift from Professor A. Tobin, UCLA, USA).

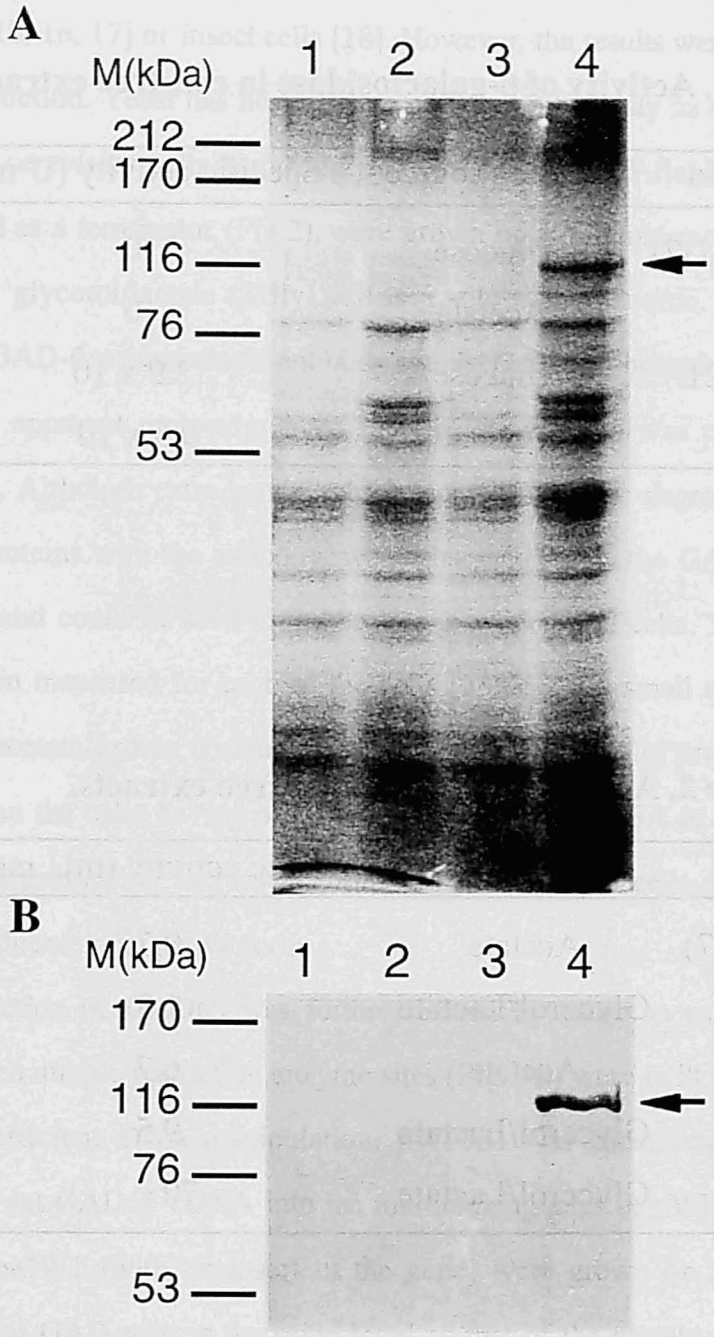

Fig. 4. SDS-PAGE (10 % acrylamide) (A) and Western blot analysis (B) of cell-free extracts (100 μg) for the detection of the expressed β-galactosidase. Coomassie brilliant blue was used for staining in A. A and B; *Lanes 1 and 2*, pMT34(-G7)-transformed cells (no insert, control); *lanes 3 and 4,* pMIZ21-transformed cells; *lanes 1 and 3*, glucose-grown cells; *lanes 2 and 4*, acetate-grown cells. Arrows represent the recombinant β-galactosidase proteins.

Table 1. Activity of β-galactosidase in cell-free extracts.

Plasmid	Carbon source	Specific activity (U mg^{-1})
pMT34(-G7)	Glucose	0
	Acetate	0
pMIZ21	Glucose	2.9 x 10
	Acetate	9.5 x 10^3

Table 2. Activity of GAD in cell-free extracts.

Plasmid	Carbon source	Specific activity (mU mg^{-1})
pMT34(-G7)	Acetate	0.75
pMW1	Glycerol/Lactate	1.3
pGAD11	Acetate	6.7
	Glycerol/Lactate	9.5
pWIG31	Glycerol/Lactate	70

GAD catalyzes the decarboxylation reaction of L-glutamate to form γ-aminobutyric acid (GABA), an inhibitory neurotransmitter. Several attempts were made to express GAD65 in bacterial cells [10, 16, 17] or insect cells [18]. However, the results were not satisfactory for a large scale production. Yeast has never been employed previously as a host for this purpose. *Saccharomyces cerevisiae* cells harboring pGAD11, in which 3'-flanking region of GAD65 cDNA was used as a terminator (Fig.2), were grown on a minimal medium containing either acetate (SA) or glycerol/lactate (SGlyLac) as a sole carbon source. Western blot analysis visualized with GAD-6, a monoclonal antibody against GAD65, showed that an immunoreactive protein with an apparent molecular mass identical to GAD65 was present in the cell-free extracts (Fig. 5). Although extra bands, which seemed to be the degradation products and/or cross-reactive proteins with the antibody, were observed below the GAD65 protein band. No corresponding band could be seen in the acetate-grown control cells. The enzymatic activity of GAD was then measured for each of the cells (Table 2). A small amount of activity was detected in the acetate-grown control cells due to GAD originally present in *Saccharomyce scerevisiae*. When the cells having pGAD11 were cultivated in SA or SGlyLac medium, the specific activity significantly increased, compared to the control cells, indicating recombinant GAD65 was produced in an active form.

The production of GAD65 was further refined by using an expression vector pWI3 (Fig.3A), in which unique restriction enzyme sites (Fig. 3B) were built between *UPR-ICL* and *TERM-ICL* for efficient DNA manipulation. pWIG31 was constructed by introducing the coding region of rat GAD65 cDNA into the multicloning sites of pWI3 (Fig. 6A). When the cells harboring pMW1 (with no insert of the gene) were grown on SGlyLac medium, the specific activity of GAD was as the same level as that of the acetate-grown cells harboring pMT34(-G7). On the other hand, a high intracellular activity of GAD could be detected when the cells harboring pWIG31 were cultivated in SGlyLac medium (Table 2). This specific activity was four times higher than that expressed in the baculovirus-insect expression system (Mauch et al. 1993b). Western blot analysis showed that recombinant GAD65 protein was surely produced in the cells (Fig. 6B).

The cells having pWIG31 showed seven to ten times higher specific activity in comparison with the cells harboring pGAD11. This fact reflects the difference in transcriptional termination

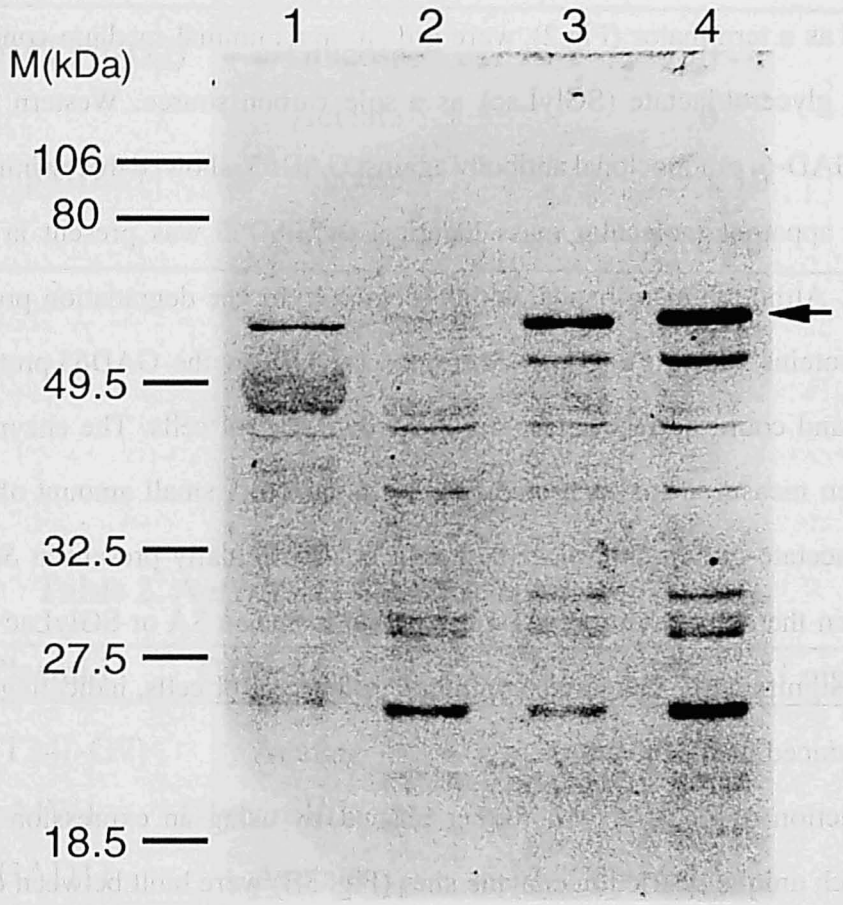

Fig. 5. Western blot analysis of the recombinant GAD65 from the cells harboring pGAD11. An aliquot (50 µg) of each cell-free extract was applied on SDS-PAGE. A GAD-6 monoclonal antibody was used for the detection of recombinant GAD65. *Lane 1*, rat brain homogenate; *lane 2*, pMT34(-G7)-transformed cells (no insert, control); *lanes 3 and 4*, pGAD11-transformed cells; *lanes 2 and 3*, acetate-grown cells; *lane 4*, glycerol/lactate-grown cells. An arrow shows the recombinant GAD65.

efficiency between these two plasmids, where pWIG31 has *TERM-ICL* and pGAD11 has the 3'-flanking region of GAD65 cDNA. In the analysis of mRNA of the respective cells by Northern blot hybridization, a clear main band was observed for the cells with pWIG31, whereas a broad band extending above the main band was observed for the cells with pGAD11 (Fig. 7). The results indicate a poor transcriptional termination efficiency in the latter case. This fact showed that 328-bp long *TERM-ICL* was sufficient for transcriptional termination.

DISCUSSION

The results mentioned here showed that *UPR-ICL* was effective for the expression of heterologous genes in *Saccharomyces cerevisiae*. Moreover, the expression vector pWI3 was useful for constructing the expression plasmid. As acetate is a relatively inexpensive inducer, the use of *UPR-ICL* is advantageous for large scale cultivation. Furthermore, transcription under the control of *UPR-ICL* is tightly repressed by glucose in *Saccharomyces cerevisiae*, and enhanced by non-fermentable carbon sources like acetate. Induction ratio (acetate/glucose) reached about 300-fold when β-galactosidase was produced. This induction ratio is relatively high among the promoters widely used for heterologous gene expression in *Saccharomyces cerevisiae*. Further investigation on the optimization of the total productivity and growth rate in connection with the induction ratio should be necessary for industrial applications.

Two isoforms (GAD65 and GAD67) are found for GAD which localizes in brain as well as in pancreatic β-cells in mammals. Attention has been focused on GAD65, since recent studies indicate that GAD65 is the major target of autoantibody associated with insulin-dependent diabetes mellitus (IDDM) [19]. Since the autoantibody appears in patient's blood prior to the development of IDDM, it is predicted that large supply of GAD65 could serve as a diagnostic tool for IDDM with high sensitivity and selectivity [20]. Therefore, an efficient production of the GAD65 protein would lead to benefits in both the fields of enzymology and clinical chemistry.

For the production of the recombinant GAD65 protein, Mauch et al. [18] emphasized the superiority of the baculovirus-insect expression system over the bacterial expression system on the point that posttranslational modification of the GAD protein would be possible,

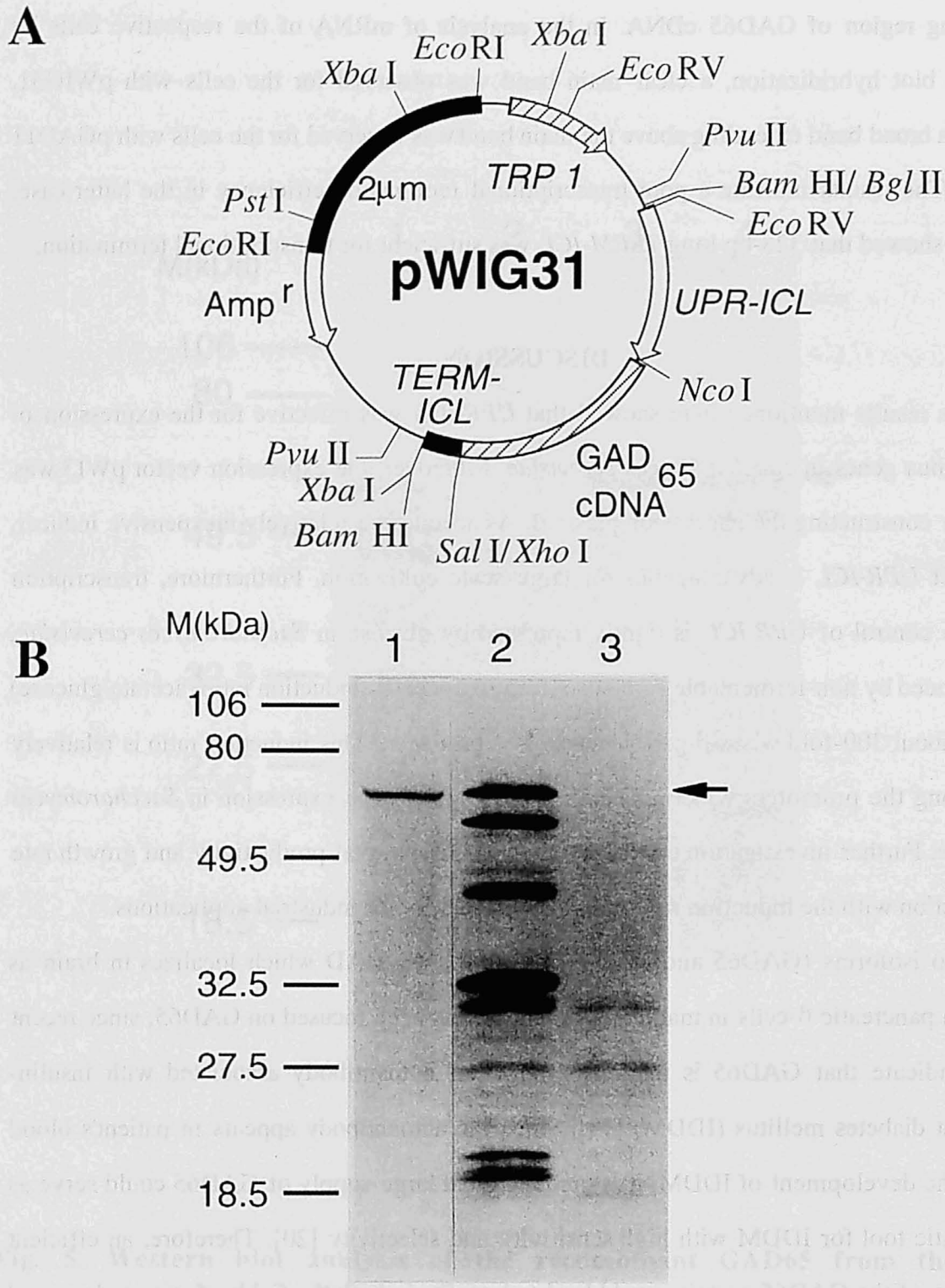

Fig. 6. Construction of the plasmid pWIG31 (A) for the expression of GAD65 cDNA and Western blot analysis of the recombinant GAD65 produced by the cells harboring pWIG31 (B). In B, an aliquot (100 µg) of each cell-free extract was applied and the same antibody (in Fig. 5) was used. *Lane 1*, rat brain homogenate; *lane 2*, pWIG31-transformed cells; *lane 3*, pMW1-transformed cells (no insert, control); *lanes 2 and 3*, glycerol/lactate-grown cells. *Arrow* represents expressed GAD65.

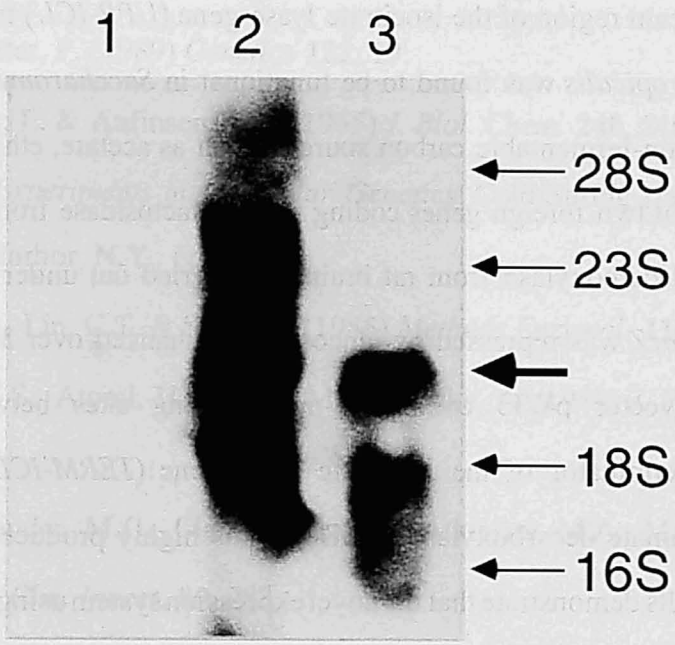

Fig. 7. Northern blot analysis of mRNA of GAD65. Total RNA (5μg) from the acetate-grown cells was used. *Lane 1*, pMW1-transformed cells (no insert, control); *lane 2*, pGAD11-transformed cells; *lane 3*, pWIG31-transformed cells. A thick arrow shows the transcribed mRNA of GAD65.

where the recombinant protein is similar to the native one. As yeast is also a eukaryotic organism, the system described in this chapter maintains these advantages. Moreover, the expressed level of GAD65 was four fold higher in pWIG31-transformed *Saccharomyces cerevisiae* than in the baculovirus-mediated insect cells. Comparing with insect cells, scale-up cultivation of yeast is far easier and inexpensive. This also makes yeast an attractive host for the large scale production of recombinant GAD65.

SUMMARY

The upstream region of the isocitrate lyase gene (*UPR-ICL*) from *n*-alkane-assimilating yeast *Candida tropicalis* was found to be functional in *Saccharomyces cerevisiae* as a novel promoter with non-fermentable carbon sources, such as acetate, ethanol, and glycerol/lactate. The expression of two foreign genes coding for β-galactosidase from *Escherichia coli* (*LacZ*) and glutamate decarboxylase from rat brain was carried out under the control of *UPR-ICL*. Expression of *LacZ* was repressed by glucose and enhanced over 300-fold by acetate. When an expression vector pWI3 containing multicloning sites between *UPR-ICL* and the transcriptional terminator of the isocitrate lyase gene (*TERM-ICL*) was used, the smaller isoform of glutamate decarboxylase (GAD65) was highly produced as a soluble and active form. These results demonstrate that the novel expression system using *UPR-ICL* and *TERM-ICL* from *Candida tropicalis* is useful for the production of heterologous proteins in *Saccharomyces cerevisiae*.

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Chapter 2. Expression of human histidine decarboxylase in *Saccharomyces cerevisiae*

INTRODUCTION

Mammalian histidine decarboxylase (HDC) catalyzes decarboxylation reaction of L-histidine to produce histamine, a neurotransmitter for the central nervous system as well as a mediator for allergic responses and gastric acid secretion. Three neurotransmitter forming decarboxylases, HDC, glutamate decarboxylase, and 3,4-dihydroxyphenylalanine (DOPA) decarboxylase, exhibit high sequence homology to suggest that these decarboxylases belong to the same family. The structural homology may suggest the common catalytic machinery among these decarboxylases; however, none of the three dimensional structure of decarboxylases has been determined. In case of HDC, due to the limited amount present in mammalian cells, the progress of HDC studies has been hampered. Several attempts to express HDC using *in vitro* expression system including *Escherichia coli* system, were either unsuccessful or unsatisfactory since the expressed protein is often insoluble and non-functional or economically unfeasible. Expression of the recombinant HDC in Sf9 insect expression system was reported for rat and human enzymes; however, the problem remained as HDC being in the insoluble fraction [1, 2]. An expression system suitable for a larger scale preparation should be designed.

Yeast *Saccharomyces cerevisiae* has been used for heterologous gene expression [3]. Yeast grows rapidly, produces high-density cell population, utilizes inexpensive media and the gene manipulation technique which is basically similar to *Escherichia coli*, and gives the typical eukaryotic-like posttranslational modifications. Recent advancement in gene manipulation has allowed us to express a number of mammalian proteins in yeast cells [3]. The author has expressed 65 kDa isozyme of glutamate decarboxylase (GAD65) in *Saccharomyces cerevisiae*, where a strong *UPR-ICL* originated from *Candida tropicalis* is employed, as described in Part I, Chapter 1. In this system, the soluble form of GAD65 was produced, which was confirmed by Western blot analysis. In this chapter, the author describes an efficient yeast expression system for HDC production.

MATERIALS AND METHODS

Chemicals

Yeast extract and peptone were purchased from DIFCO. Taq DNA polymerase (*Thermus aquaticus* YT1) was obtained from Wako Pure Chemical (Osaka, Japan).

Strains

Escherichia coli DH5α was used as a host for recombinant DNA manipulation. *Saccharomyces cerevisiae* strain MT8-1 (*MATa*, *ade*, *his3*, *leu2*, *trp1*, *ura3*) [4] was used as a host for the protein production.

Construction of the plasmid (pWIH31) for expressing HDC cDNA

The expression vector, pWI3, used for the expression of rat glutamate decarboxylase cDNA as described in the previous chapter, was employed for the HDC expression. Primers, 5'-AGACCATGGAGCCTGAGGAGTAC-3' and 5'-CCTCGAGTCTAAACCATAGCCTGC-AG-3', were designed to introduce *Nco*I and *Xho*I sites at the initiation codon and termination codon, respectively. The amplification was performed on a GeneAmp PCR System 9600, Perkin Elmer Applied Biosystems (Foster City, CA, U.S.A.), with 25 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 2.5 min) using Taq DNA polymerase. Products were digested with *Nco*I and *Xho*I and inserted into the multiple cloning site of pWI3 (Fig. 1) to produce pWIH31. The correct insertion of the HDC cDNA was confirmed by DNA sequencing.

Cultivation and preparation of cell-free extract

Yeast cell cultivation and cell-free extract preparation were carried out basically as described in the previous chapter. Pre-cultivation was carried out in the medium containing 1% yeast extract, 1% peptone, and 2% glucose (YPD medium). A large-scale cultivation, usually around 10L, was carried out in a medium containing 0.67% yeast nitrogen base without amino acid, 0.002% histidine, 0.003% leucine, 0.002% adenine sulfate, 0.002% uracil, and 2% sodium acetate. All cultivations were performed at 28 °C.

Cells in the late-exponential phase were harvested by centrifugation and washed twice with distilled water. Cells were suspended in the lysis buffer, consisted with 35 μg/ml phenylmethylsulfonyl fluoride (PMSF), 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM 2-aminoethylisothiouronium bromide (AET),

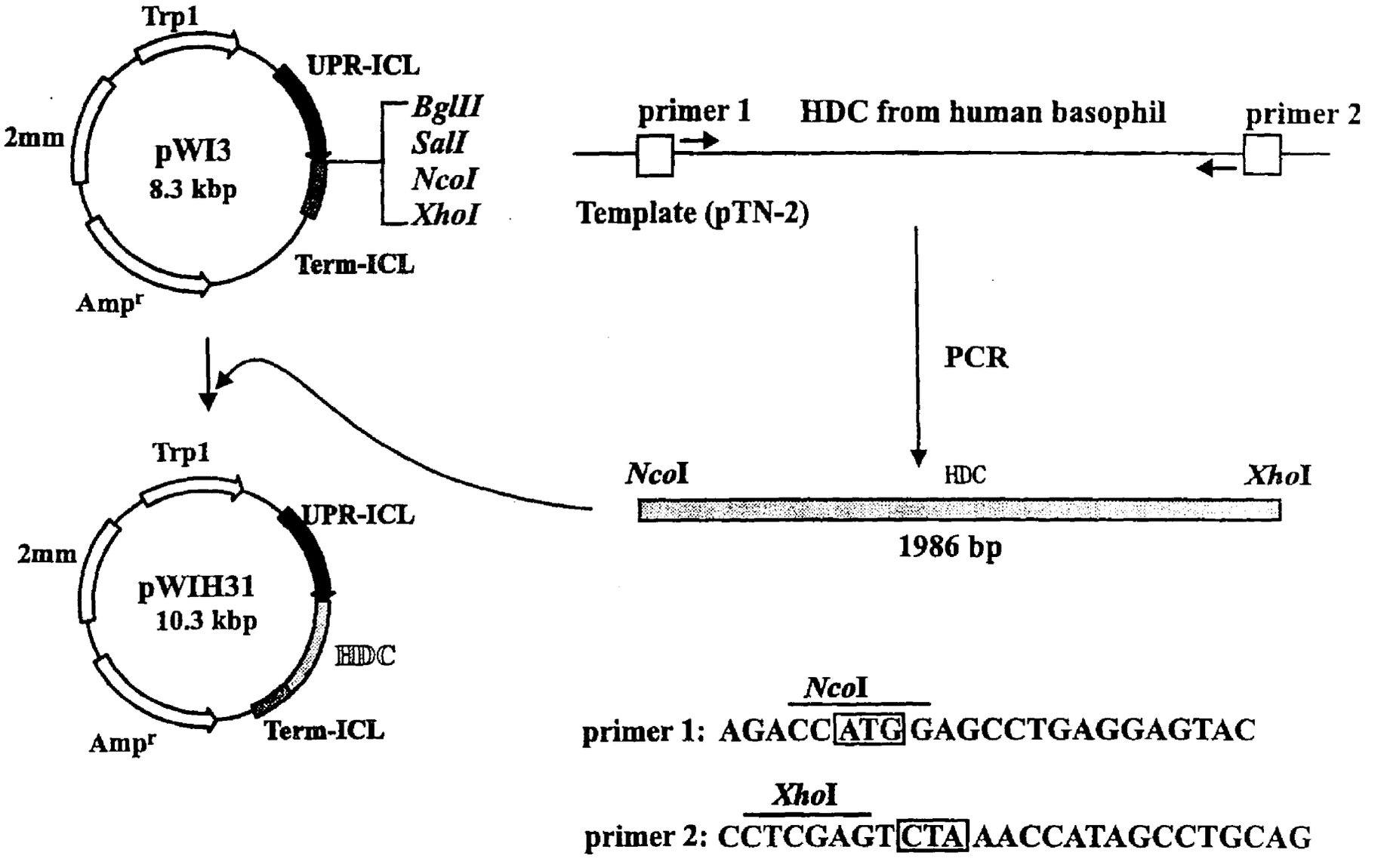

Fig. 1. Structure and construction of recombinant expression vector, pWIH31. Human basophil-originated HDC cDNA was used as a template. After PCR and *Nco*I and *Xho*I digestion, 1986 bp-long fragment was inserted into pWI3 to give pWIH31. Shaded area indicates HDC coding region and solid arrow area represents *UPR-ICL*.

0.1 mM dithiothreitol (DTT), and 0.5 mM pyridoxal 5'-phosphate (PLP) in 50 mM sodium phosphate buffer (pH 7), then, were disrupted by vortexing with glassbeads. One min each of the disruption was repeated three times to ensure the complete cell breakage, as judged by microscopic examination. Between the disruption cycle, 3 min cooling periods were set to ensure the efficient cooling. Centrifugation of the cell homogenate at 10,000 *g* for 30 min gave a supernatant used as cell-free extract.

Assay

Histidine decarboxylase activity was assayed essentially as described by Ohmori *et al.* [5]. The 1 ml assay mixture contained 0.2 mM dithiothreitol, 0.01 mM pyridoxal 5'-phosphate, 2% of polyethylene glycol #300 in 0.1 M potassium phosphate buffer, pH 6.8 and enzyme solution. It was pre-incubated at 37 °C for 5 min. The reaction was initiated by adding 0.8 mM L-histidine and terminated by adding 40 μl of 60% perchloric acid. After the brief centrifugation, supernatant was neutralized by adding 0.5 M sodium phosphate buffer, pH 6.5, while pH being monitored by the change of bromophenol blue color from yellow to green. The neutralized assay mixtures were placed on columns made of disposable Pasteur pipettes packed with Amberlite CG-50 (Na^+ form). Proteins and other unbound materials were washed off initially with distilled water and histidine was eluted off with 0.1 N HCl wash. Histamine formed during the incubation was eluted off from the column with 1.5 ml of 0.5 N HCl. The eluents were immediately neutralized with 0.125 ml of 5 N NaOH, added with 50 μl of *o*-phthaldialdehyde (OPA; 1% in methanol), and left for 5 min at room temperature. After adding 0.125 ml of 6 N HCl, fluorescence intensity was measured to quantitate the amount of OPA derivative on a Shimazdu RF-5300PC spectrofluorophotometer (Kyoto, Japan) with excitation wavelength of 355 nm and emission wavelength of 440 nm. Fluorescence intensity was calibrated with standard histamine solution and intensity values obtained were converted to the amount of histamine formed per incubation period.

Protein assay

Protein amount was determined using BCA assay, Pierce Chemical (Rockford, IL, U.S.A.), with bovine serum albumin as the standard.

SDS-PAGE

Polyacrylamide gel electrophoresis in the presence of SDS was carried out on 10% slab

gels [6]. Proteins were stained with Coomassie brilliant blue and destained with 20% methanol in 10% acetic acid solution. BioRad prestained low-range molecular markers (Hercules, CA, U.S.A) were used as molecular mass standard.

RESULTS

The *Saccharomyces cerevisiae* cells harboring pWIH31 or pWI3 plasmid were pre-cultivated overnight at 28 °C in YPD medium and transferred to a minimum medium containing sodium acetate as an inducer. Cells were harvested at various time points and their populations were measured by turbidity at 660 nm (Fig. 2). *Saccharomyces cerevisiae* cells showed linear growth for 25 h and then reached the stationary phase, where the turbidity of medium remained slightly above 1.0. *Saccharomyces cerevisiae* strain carrying pWIH31 or pWI3 plasmid showed identical growth. After being harvested, the cells were disrupted with glassbeads, and soluble and insoluble fractions were obtained by centrifugation. Protein concentration and HDC activity were estimated for the soluble fractions (Fig. 2). The increase in the total amount of protein was proportional to the cell population.

HDC activity was measured directly from the aliquots of cell extracts (Fig. 2). HDC activity increased rather rapidly, having the maximum activity around 25 h of the induction. Induction of 25 h was employed for a large-scale HDC preparation. The typical specific activity obtained with the crude cell extracts is shown in Table 1. Yeast cells harvested at 25 h gave 2.5 to 4 g of wet cell paste per liter of medium. Protein determination of the total soluble protein indicated that 15 mg protein was present per g of the wet cell paste. Assuming that pure HDC has the specific activity of 800 nmol min^{-1} mg^{-1} as described by Ohmori *et al.* [5], the amount of HDC in the crude protein extract should be around 0.05%. Therefore, the total HDC protein present in the cell lysate is estimated around 20 to 30 μg per liter of growth medium.

For those proteins with the expression level of 0.05%, it is reasonable to assume that the protein band in SDS-PAGE analysis cannot be visualized. As expected, an SDS-PAGE analysis failed to visualize the protein band corresponding to HDC. Although it was preliminary, Western blot analysis performed by Dr. Yatsunami at Japan Tobacco. Inc., with anti-HDC antibody gave a positive band, apparently, corresponding to 74 kDa form (private communication).

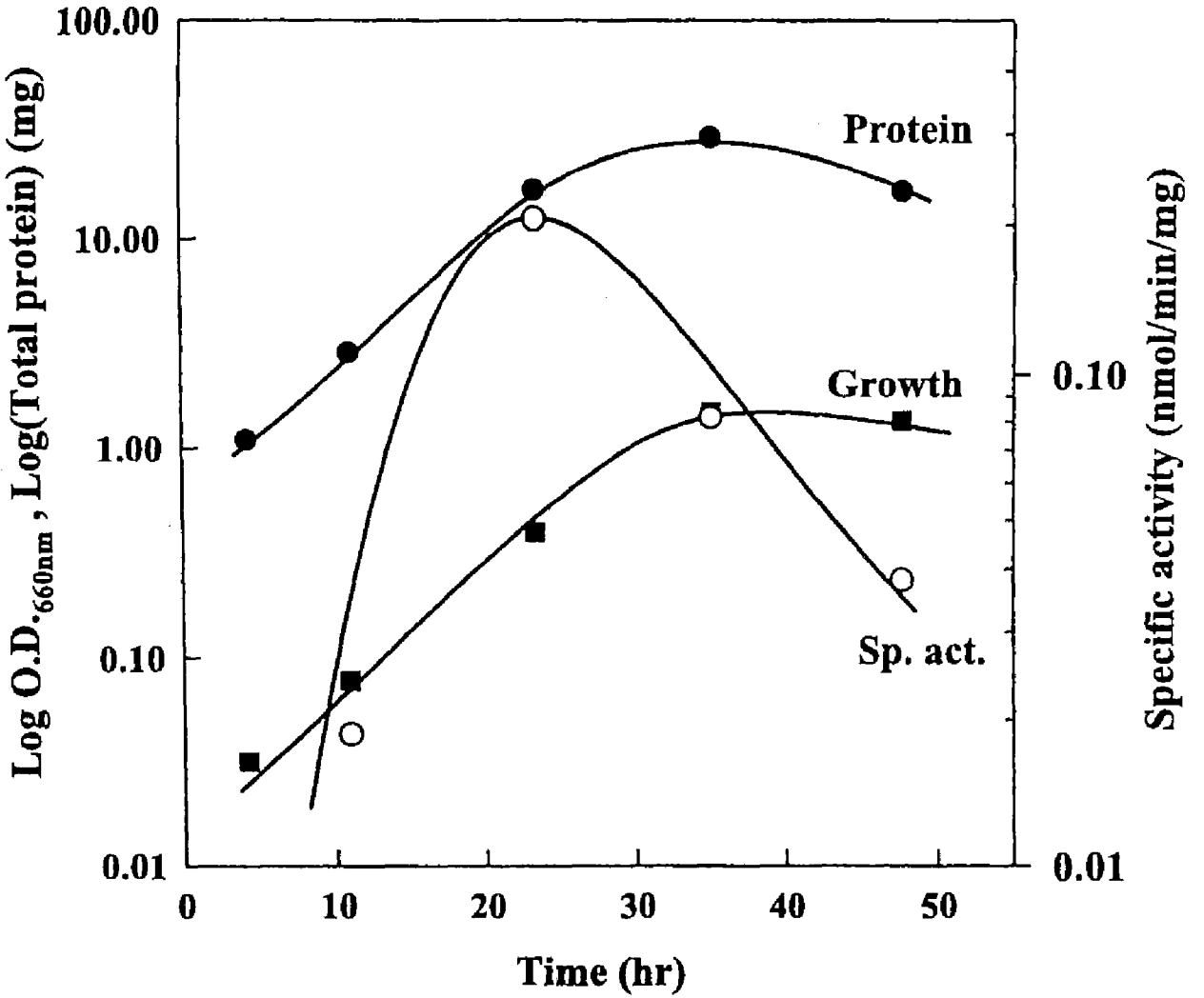

Fig. 2. Growth curve of *Saccharomyces cerevisiae* carrying pWIH31. Cell density at 660 nm, total protein (mg) in cell lysate and specific activity of HDC in cell lysate are shown with ■, ●, and ○, respectively.

Table 1. HDC activities from various tissues and expression systems.

Tissue or cell origin	Specific activity (pmol min^{-1} mg^{-1})	Reference
Rat Brain	0.76	[7]
Heart	0.0	[7]
Lung	0.07	[7]
Liver	0.01	[7]
Stomach	10	[7]
Spleen	0.13	[7]
Kidney	0.0	[7]
Mast cells	118	[7]
Whole fetus	17	[7]
Rat Mast cells	20	[8]
Mastocytoma P-815	64	[5]
Sf9	2700	[1]
Yeast pWIH31	210	This study
pWI3	0.0	This study

DISCUSSION

The author has shown that recombinant human HDC is expressed as a soluble form in yeast *Saccharomyces cerevisiae*. The relatively large-sized proteins are known for their difficulty in expressing as a soluble form in bacterial expression systems. *Saccharomyces cerevisiae* expression system under the control of *UPR-ICL* is utilized since it has shown to express a relatively large sized mammalian protein, 65 kDa GAD, as described in PartI, Chapter1. The yeast expression system is suited for a scale-up of the recombinant protein production. The expression system reported here is also economically sound, since the costs for growth media and an inducer such as acetate are low.

Protease sensitive proteins such as HDC [7] should be avoided from any exposure to possible proteolytic digestion. Yeast is a known source for proteases, thus, a possible effect of proteolysis should be considered. This problem was overcome by the addition of a mixture of protease inhibitors. In the case described in this chapter, the presence of the mixture, PMSF, pepstatin A, leupeptin, and EDTA, allowed prolonged storage of crude cell lysate for several days at 4 °C without altering any HDC activity. The low temperature is maintained as to stop the unnecessary cell breakage which leads to the undesired damage on proteins. The breakage of yeast cells was initially accomplished by an ultrasonic treatment in the glutamate decarboxylase expression work in the previous chapter. However, for the more efficient breakage of yeast cells, later experiments have utilized glassbeads method.

The level of HDC expression in *Saccharomyces cerevisiae* system is satisfactory based on the values obtained for tissue samples. For instance, the specific activities of HDC isolated in rat mast cell, rat whole fetus, and rat stomach are 60-120, 17, and 10 (pmol min^{-1} mg^{-1}), respectively (Table 1). The present yeast expression system has given specific activity of 210 (pmol min^{-1} mg^{-1}), twice of that for mast cells. This level of expression is, on the other hand, considerably lower than that of Sf9 insect cell system [1]; the cell extract of Sf9 exhibits 2.7 (nmol min^{-1} mg^{-1}) of specific activity but it is expensive for scale-up. Since the current facility for yeast cultivation allows the scale-up from 1L to 100L cultivation level, the yeast expression system is an efficient method for protein structural studies.

It has been questioned whether or not the soluble HDC form expressed in *Saccharomyces cerevisiae* system is 74 kDa. Ohmori *et al.*[5] reported that the 74 kDa protein, a precursor enzyme, was the insoluble form in the Sf9 system and that the mature enzyme, the 53 kDa

protein and C-terminal segment processed, was produced as a soluble form. Although HDC should be purified to be conclusive, the soluble protein in the yeast expression system is likely to be the 74 kDa protein. It should be noted that 74 kDa of human HDC is enzymatically active [2].

SUMMARY

Histidine decarboxylase cDNA (1,986-bp) from human basophilic leukemia cell line, KU-812-F, was inserted into a shuttle vector carrying the isocitrate lyase gene promoter (*UPR-ICL*) and terminator originated from *Candida tropicalis*. The plasmid was introduced into a *Saccharomyces cerevisiae* host strain, which was cultivated in the medium containing acetate. Histidine decarboxylase activity in the cell lysate was measured throughout the incubation period where the maximum histidine decarboxylase activity was obtained at around 25 h, near the end of the exponential growth phase. A typical preparation gave the specific activity of 210 (pmol min^{-1} mg^{-1}) in the crude cell lysate. This value was twice and three times higher than that in the crude extract of mast cells and in mastocytoma P-815 culture cells, respectively. The enriched activity of histidine decarboxylase in the soluble fraction suggests that yeast expression system provides the properly folded and catalytically active enzyme. Therefore, the present expression system is suitable for the structural studies including X-ray crystallography, which require a large amount of the enzyme.

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Chapter 3. Expression of recombinant thermostable cycloinulo-oligosaccharide fructanotransferase in *Saccharomyces cerevisiae*

INTRODUCTION

Cycloinulo-oligosaccharides (cyclofructans [CF]) are the cyclic oligosaccharides which consist of six to eight molecules of β-(2→1)-linked D-fructofranose (CF6, CF7, and CF8). This bowl-shaped structure of CF resembles that of cyclodextrins (cyclic malto-oligosaccharides), which have been used extensively in medical, food, and chemical fields [1]. This mainly owes to the ability of cyclodextrins to form inclusion complexes with a wide variety of guest molecules [1]. An analysis of the crystal structure of CF6 revealed that its central skeleton had the same structure as that of 18-crown-6 crown ether [2, 3]. Accordingly, CF6 has the ability to form complexes with metal cations, which is the characteristic of crown ethers [4-6]. The practical application of CF has been widely examined, and it has been shown that CF has stabilizing effects on various materials during freezing and thawing, e.g., dough used for bread, liposomes used in the cosmetic and medical fields, and so on. Moreover, an alkylated-form of CF can be used as a new alternative to crown ethers, indicating the possibilities of its application in chemical industry.

CF is synthesized from inulin through an intramolecular transfructosylation reaction. Inulin is a polyfructan consisting of a linear β-(2→1)-linked polyfructose chain with a terminal glucose residue. The enzyme which catalyzes this transfructosylation is cycloinulo-oligosaccharide fructanotransferase (CFTase). At present, two microorganisms, *Bacillus circulans* OKUMZ 31B [2] and *Bacillus circulans* MCI-2554 [7], have been found to produce CFTase. Amino acid sequences were analyzed for peptide fragments of the CFTase of *Bacillus circulans* MCI-2554, showing that one fragment contained a similar amino acid sequence with the enzymes hydrolyzing β-(2→1) glycosidic linkage (invertase, sucrase, inulinase or levanase) [8].

The CFTase gene from *Bacillus circulans* MCI-2554 was isolated and its nucleotide sequence was determined (GenBank/EMBL/DDBJ accession number: D87672). In future uses of CF for drugs or foods, the choice of the host organism for CFTase production is critical. For example, *Escherichia coli* is not suitable because of its toxic cell wall pyrogens.

Bakers' yeast, *Saccharomyces cerevisiae*, a eukaryote having a GRAS ('generally recognized as safe') status, is a suitable host for safe production of foreign proteins [9, 10]. The author has developed a novel and powerful heterologous gene expression system in *Saccharomyces cerevisiae* using the 5'-upstream region of isocitrate lyase gene (*UPR-ICL*) from an *n*-alkane-assimilating yeast *Candida tropicalis*, as described in Part I, Chapter 1. *UPR-ICL*-mediated transcription is repressed by glucose, but highly induced by non-fermentable carbon sources such as glycerol, acetate or ethanol [11, 12] and *UPR-ICL* is shown to be as powerful a promoter in *Saccharomyces cerevisiae* as *GAL1* or *GAL7* [11].

In this chapter, the author reports the expression of a truncated fragment of the CFTase gene of *Bacillus circulans* MCI-2554 in *Saccharomyces cerevisiae* under the control of *UPR-ICL* and purification and characterization of the recombinant protein. Recombinant CFTase protein produced by *Saccharomyces cerevisiae* (*Sc*CFTase) was efficiently secreted in the culture supernatant in an active form. Furthermore, stable production and secretion of *Sc*CFTase were attained by constructing a *Saccharomyces cerevisiae* strain in which the CFTase genes were integrated into its chromosomes.

MATERIALS AND METHODS

Strains

Escherichia coli DH5α was used as a host for recombinant DNA manipulation. *Saccharomyces cerevisiae* strain W303-1A (*MAT***a** *ade2-1 his3-11 leu2-3,112 trp1-1 ura3-1 can1-100*) was used as a host for the protein production. Transformation of yeast was performed by the lithium acetate method [13].

Construction of vectors

The prepro secretion sequence of the α-factor was amplified from plasmid pLS01 by PCR with the following primers: 5'-AAGGATCCGTCGACATGAGATTTCCTTCAATT-3' and 5'-TCTCTAGAATCCAAAGATACCCCTTC-3'. pLS01 has 1.7 kbp *Eco*RI-*Eco*RI fragment of *MFα1* inserted at *Eco*RI site of pUC13 [14]. The amplified fragment was cut with *Sal*I and *Xba*I and inserted into pUC19, which has *Xho*I site instead of *Sma*I site. The CFTase gene was amplified from pECF21ΔK21, which contains the ΔK2 fragment of CFTase gene with the following primers: 5'-CGGAATCTCGTCTAGAGCCGAACCCG-3', and 5'-CGAGTCGAGACTCGAGATCTAAGCTT-3'. The ΔK2 fragment is a truncated fragment of CFTase gene which lacks the region encoding the three N-terminal repeat sequences (residues

57 to 558). The amplified fragment was cut with *Xba*I and *Xho*I and placed at the downstream of α-factor prepro secretion sequence. The *Xba*I site located between α-factor prosequence and CFTase gene corresponds to the last two amino acids of α-factor prosequence. Introduction of the *Xba*I site changed its corresponding amino acid sequence, Lys-Arg to Ser-Arg, and Lys-Arg is the recognition sequence of Kex2 protease for cleavage of the α-factor prosequence. Therefore, site-directed mutagenesis was performed to revert the nucleotide sequence. After site-directed mutagenesis, the *Sal*I-*Xho*I fragment containing α-factor prepro sequence and ΔK2 fragment of the CFTase gene was introduced into pW13 (described in Part I, Chapter 1) to make pWIF31. For the construction of chromosome integrating vectors, a *Pvu*II-*Pvu*II fragment from pWIF31 containing *UPR-ICL*, α-factor prepro sequence, ΔK2 fragment of CFTase gene, and the 3'-noncoding region of the isocitrate lyase gene of *Candida tropicalis* (*TERM-ICL*) was excised, and introduced into yeast integrative vectors pRS403 and pRS404 [15] digested by *Pvu*II, to construct pWIF33 and pWIF34, respectively. pWIF33 and pWIF34 were both cut by *Bst*XI, and introduced into *Saccharomyces cerevisiae* strain W303-1A to construct CF/HW2A strain. In pWIF33 or pWIF34, there is one *Bst*XI site located within *HIS3* or *TRP1*, respectively.

Purification of *Sc*CFTase from culture supernatant

Yeast cells harboring pWIF31 were cultivated in SA medium (0.67% yeast nitrogen base without amino acid (Difco, Detroit, MI, USA), 1% sodium acetate, and appropriate supplements) at 30°C until the early stationary phase (60 h). Culture broth was then centrifuged to remove yeast cells. The remaining culture supernatant was ultrafiltrated by a Diaflo-membrane filter YM-10 (Amicon, Beverly, MA, USA). The concentrated enzyme solution was applied to a DEAE-Sepharose CL-6B column (Pharmacia, Uppsala, Sweden) equilibrated with 67 mM KPB (pH 6.9). The absorbed enzyme was eluted with a linear gradient of 0.2M to 0.3M NaCl. At low salt concentration, *Sc*CFTase 1 (116kDa) was first eluted from the column, followed by *Sc*CFTase 2 (117kDa) and *Sc*CFTase 3 (116kDa).

Electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method reported by Laemmli [16]. The molecular masses were estimated by using the following standard proteins (Pharmacia) : myosin, 212 kDa; α_2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa; glutamate dehydrogenase, 53 kDa.

N-terminal amino acid sequence analysis

After SDS-PAGE, proteins were transferred to a sheet of ProBlott (Applied Biosystems, Foster City, CA, USA). The filter was set in a protein sequencer 610A (Applied Biosystems) and analyzed as recommended by the vendor.

Endoglycosidase H digestion

*Sc*CFTase was digested with endoglycosidase H (Endo H_f) (New England BioLabs, Beverly, MA, USA) according to the description given by the supplier.

Enzyme assay

CFTase activity was assayed as described Kushibe *et al.* [8]. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of cycloinulohexaose (CF6) per minute. For an easier method to check CFTase activity, assay mixture was analyzed by TLC. Reaction mixture was spotted on TLC plate (F254) (Merck, Darmstadt, Germany), and then plate was developed with 1:1:4 (vol/vol/vol) water/1-butanol/2-propanol. Sugars were detected with naphthoresorcinol/H_2SO_4 reagent.

Southern blot analysis

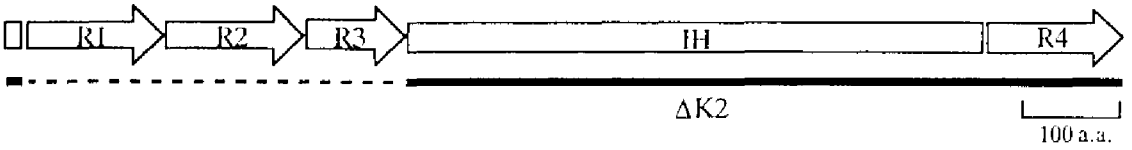
Southern blot analysis was carried out as described Kurihara *et al.* [17] using a biotin-labeled probe prepared with BioNick labeling system (Bethesda Research Laboratories Life Technologies (BRL), Gaithersburg, MD, USA). Detection was carried out using Photo-Gene Nucleic Acid Detection System (BRL).

RESULTS

Construction of the plasmid for expression of a truncated form of CFTase gene.

A DNA fragment containing the CFTase gene was isolated from the genomic library of *Bacillus circulans* MCI-2554. Sequence analysis showed that there was a long open reading frame which encoded a protein of 1,503 amino acid residues (Fig. 1A) (GenBank/EMBL/DDBJ accession number: D87672). The overall structure of the CFTase gene showed that there were four repeat sequences, three of which located in the N-terminal region (R1 to R3), and one of which was located near the C-terminus (R4). Between these repeat sequences, there was a region whose sequence showed similarity to that of invertase (IH). For production of recombinant CFTase in *Saccharomyces cerevisiae*, a truncated fragment of the CFTase gene (ΔK2 fragment) was used which lacks the region encoding the three N-terminal repeat sequences (residues 57 to 558). When the ΔK2 fragment was expressed in *Escherichia coli*, it was

A



B

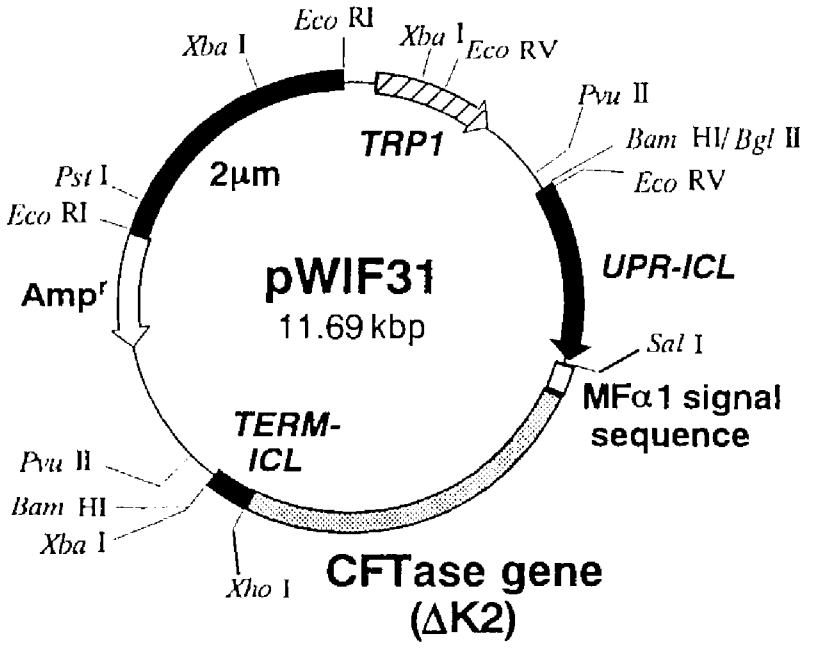


Fig. 1. Structure of CFTase gene(A) and pWIF31(B). (A) R1 to R4, regions of repeat sequence. IH, a region which has a sequence homology with invertase gene. Thick bar, the region included in ΔK2 fragment. (B) *UPR-ICL*, the upstream region of isocitrate lyase gene of *Candida tropicalis*; *TERM-ICL*, the terminator region of isocitrate lyase gene of *Candida tropicalis*; CFTase gene (ΔK2), ΔK2 fragment in (A).

Table 1. Purification of *Sc*CFTase from culture supernatant

Fraction	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Purification (-fold)
Ultrafiltration (YM10)	10.6	229	21.4	100	1.00
DEAE-Sepharose CL-6B					
*Sc*CFTase 1 (116kDa)	0.196	7.33	37.4	3.20	1.75
*Sc*CFTase 2 (117kDa)	0.454	21.1	46.5	9.21	2.17
*Sc*CFTase 3 (116kDa)	0.122	6.60	54.1	2.88	2.53

known that the recombinant protein produced had a significant enzymatic activity and that CFTase activity in the culture supernatant was even higher than when the entire CFTase gene was expressed.

For the secretion of *Sc*CFTase into the culture medium, the prepro leader sequence of the α-factor precursor (*MFα1*) was fused to the ΔK2 fragment of the CFTase gene. This was introduced into the multi-copy plasmid pWI3 (described in Part I, Chapter 1), to construct pWIF31 (Fig. 1B). The expression plasmid, pWI3, has *UPR-ICL* as a promoter and the 3'-noncoding region of the isocitrate lyase gene of *Candida tropicalis* (*TERM-ICL*) as a transcriptional terminator, along with a multi-cloning site in between. pWIF31 was then introduced into *Saccharomyces cerevisiae* strain W303-1A.

Purification of *Sc*CFTase.

When the pWIF31-transformed cells were grown on acetate, CFTase activity could be detected in the culture supernatant. Purification of *Sc*CFTase was performed using culture supernatant after cultivation for 60 h. The culture supernatant was first ultrafiltrated, and the concentrated enzyme solution was then applied to a DEAE-Sepharose CL-6B column equilibrated with 67 mM potassium phosphate buffer (pH 6.9). The absorbed enzyme was eluted with a linear gradient of 0.2M to 0.3M NaCl. The purification data are described in Table 1.

By SDS-PAGE analysis of the fractions with CFTase activity after DEAE-Sepharose column chromatography, three protein molecules with CFTase activity [*Sc*CFTase 1 (116kDa),

*Sc*CFTase 2 (117kDa), and *Sc*CFTase 3 (116kDa)] were detected (Fig. 2A; lanes 1, 3, and 5). There were only slight differences in the specific activities of the three molecules, and all of the preparations showed higher specific activities than that of the native CFTase from *Bacillus circulans* MCI-2554 (*Bc*CFTase; 36.4 U/mg) [8]. *Sc*CFTase 2 was found to be the largest in amount, being the major product in this expression system.

N-terminal amino acid sequence for each molecule was then determined (Fig. 2B). N-terminal amino acid sequences of *Sc*CFTase 2 and *Sc*CFTase 3 were identical (Ala-Glu-Pro-Gly-Ala-Asp-Ile-Glu-Asp-Ala for *Sc*CFTase 2 and Ala-Glu-Pro-Gly-Ala-Asp-Ile- for *Sc*CFTase 3) and also corresponded to the sequence immediately after the Kex2 protease cleavage site. On the other hand, *Sc*CFTase 1 had a distinct N-terminal sequence (Glu-Ala-Glu-Ser-Glu-), which started 14 amino acids from the first Ala detected in *Sc*CFTase 2 and *Sc*CFTase 3.

As there are five potential N-glycosylation sites (Asn-X-Ser/Thr) in *Sc*CFTase, each *Sc*CFTase was treated with endoglycosidase H (EndoH), an enzyme which removes N-glycosylated carbohydrate chain(s) from the protein. After EndoH treatment, differences in molecular mass were compared by SDS-PAGE. As for *Sc*CFTase 1 and *Sc*CFTase 2, decreases in the molecular masses were observed, while the molecular mass of *Sc*CFTase 3 did not change (Fig. 2A; lanes 2, 4, and 6). Moreover, the molecular mass of EndoH-treated *Sc*CFTase 2 was the same as that of *Sc*CFTase 3. Considering these results along with the N-terminal amino acid sequence of each *Sc*CFTase, the author concluded that *Sc*CFTase 1 was an artifact of *Sc*CFTase 2 due to limited proteolysis at the N-terminus and that *Sc*CFTase 3 had the same protein moiety as *Sc*CFTase 2 but did not have N-glycosylated carbohydrate chain(s).

Properties of *Sc*CFTase.

By using the purified *Sc*CFTase 2 protein, enzymatic properties with respect to pH and temperature were analyzed. Figure 3A shows the effects of pH on the activity and stability of the enzyme. The optimal pH of *Sc*CFTase 2 was pH 8.0, and over 80 % of the activity was still detected after incubation for 30 min at pH 6.0 to 10.0, as happened with *Bc*CFTase [8]. Figure 3B shows the effects of temperature on the activity and stability of the enzyme. The maximal activity was observed at 45 °C as with *Bc*CFTase. Interestingly, the *Sc*CFTase 2 showed much higher thermostability than *Bc*CFTase. A high level of activity (62.1 % of the initial activity) remained after 30 min of incubation at 70 °C, while *Bc*CFTase was reported

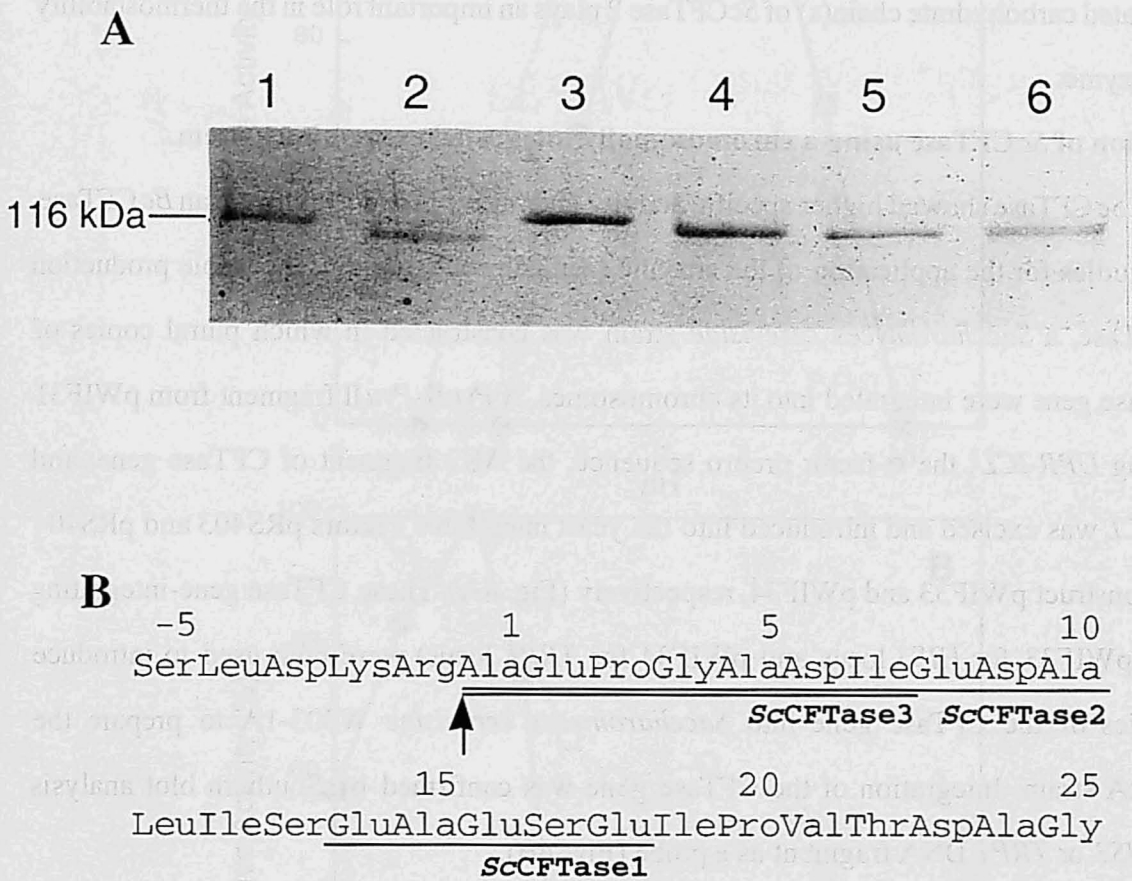


Fig. 2. SDS-PAGE analysis (7.5 % acrylamide) of *Sc*CFTase (A) and amino acid sequence at the junction of α-factor prosequence and CFTase (B). (A) *Lanes 1 and 2*, *Sc*CFTase 1; *Lanes 3 and 4*, *Sc*CFTase 2; *Lanes 5 and 6*, *Sc*CFTase 3. *Lanes 1, 3, and 5*, without Endo H treatment; *Lanes 2, 4, and 6*, with Endo H treatment. (B) Position 1 (Ala) corresponds to the first amino acid of *Sc*CFTase. *Arrow* indicates the Kex2 protease cleavage site. Amino acid sequence of each *Sc*CFTase molecules (*Sc*CFTase 1, *Sc*CFTase 2, and *Sc*CFTase 3) determined by N-terminal amino acid analysis is *underlined*.

to lose its activity under these conditions [8, 18]. Even after incubation at 80 °C or 90 °C, *Sc*CFTase 2 retained 53.6 % or 20.6 % of the initial activity, respectively. This high thermostability, however, was not observed for *Sc*CFTase 3: only 10.0 % of the initial activity was observed after 30 min of incubation at 70 °C. These results indicate that N-glycosylated carbohydrate chain(s) of *Sc*CFTase 2 plays an important role in the thermostability of the enzyme.

Production of *Sc* CFTase using a chromosomally integrated expression system.

As *Sc* CFTase showed higher specific activity and higher thermostability than *Bc* CFTase, further studies for the application of the enzyme seemed promising. For the stable production of *Sc*CFTase, a *Saccharomyces cerevisiae* strain was constructed in which plural copies of the CFTase gene were integrated into its chromosomes. A *Pvu*II-*Pvu* II fragment from pWIF31 containing *UPR-ICL*, the α-factor prepro sequence, the ΔK2 fragment of CFTase gene, and *TERM-ICL* was excised and introduced into the yeast integrative vectors pRS403 and pRS404 [15] to construct pWIF33 and pWIF34, respectively (Fig. 4A). These CFTase gene-integrating vectors (pWIF33 for *HIS3* locus and pWIF34 for *TRP1* locus) were both used to introduce two copies of the CFTase gene into *Saccharomyces cerevisiae* W303-1A to prepare the CF/HW2A strain. Integration of the CFTase gene was confirmed by Southern blot analysis with a *HIS3* or *TRP1* DNA fragment as a probe (Fig. 4B).

CF/HW2A was cultivated in a flask using YPE medium (1% yeast extract, 2% peptone, 2% [vol/vol] ethanol), and the CFTase activity in the culture supernatant was assayed periodically over 120 h. YPE medium was previously shown to highly induce *UPR-ICL*-mediated gene expression [12]. CFTase activity significantly increased between 24 and 48 h cultivation, and continued to increase gradually even until 120 h cultivation. The level of CFTase activity at this point was 391 U per liter of culture, which corresponded to 8.40 mg CFTase protein per liter of culture (Fig. 5).

The mitotic stability of the integrated exogenous DNA sequence after 120 h of cultivation was estimated by comparing the number of colonies grown on a nonselective YPD plate and that on a selective SD plate. With CF/HW2A, the number of His^+ Trp^+ colonies on the SD plate was about 94 % of that on the YPD plate. When cultivation in YPE medium was repeated five times, the number of His^+ Trp^+ CF/HW2A colonies was still over 80% of that on the YPD plate.

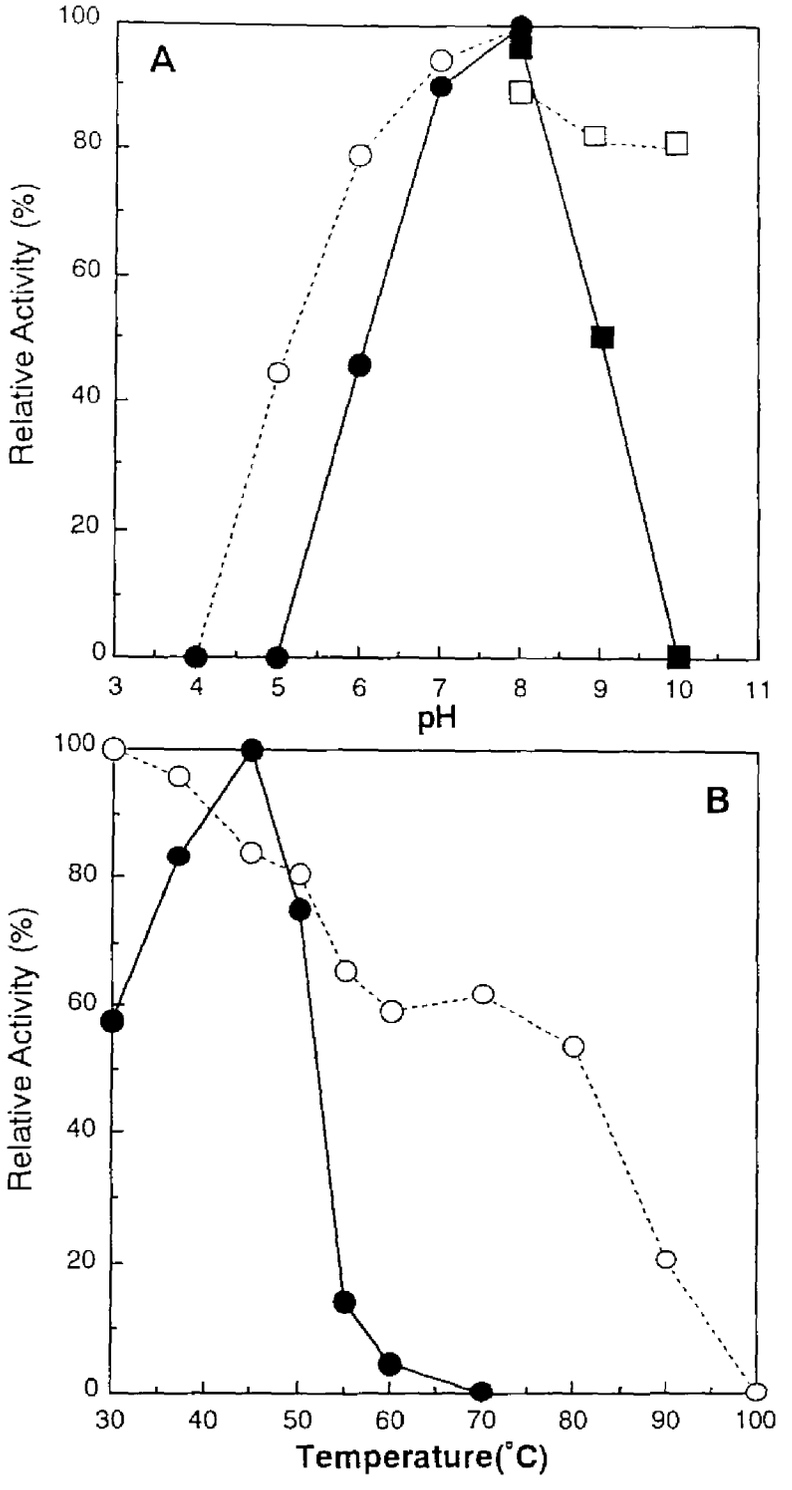


Fig. 3. Effects of pH (A) or temperature (B) on the activity and stability of *Sc*CFTase 2. (A) Enzyme activity(●, 0.1M citrate-phosphate buffer and ■, 0.1M borate buffer) was measured at 37 °C at indicated pH. pH stability of the enzyme (○, 0.16M citrate-phosphate buffer and □, 0.16M borate buffer) was measured by incubating enzyme at indicated pH for 30 min at 37°C, then residual activity was measured in 0.2M phosphate buffer (pH6.9) at 37 °C. (B) Enzyme activity (●) was measured (in 67mM phosphate buffer, pH6.9) at indicated temperatures. Thermostability of the enzyme (○) was measured by incubating enzyme at indicated temperature for 30 min in 67mM phosphate buffer (pH6.9), then residual activity was measured at 37 °C.

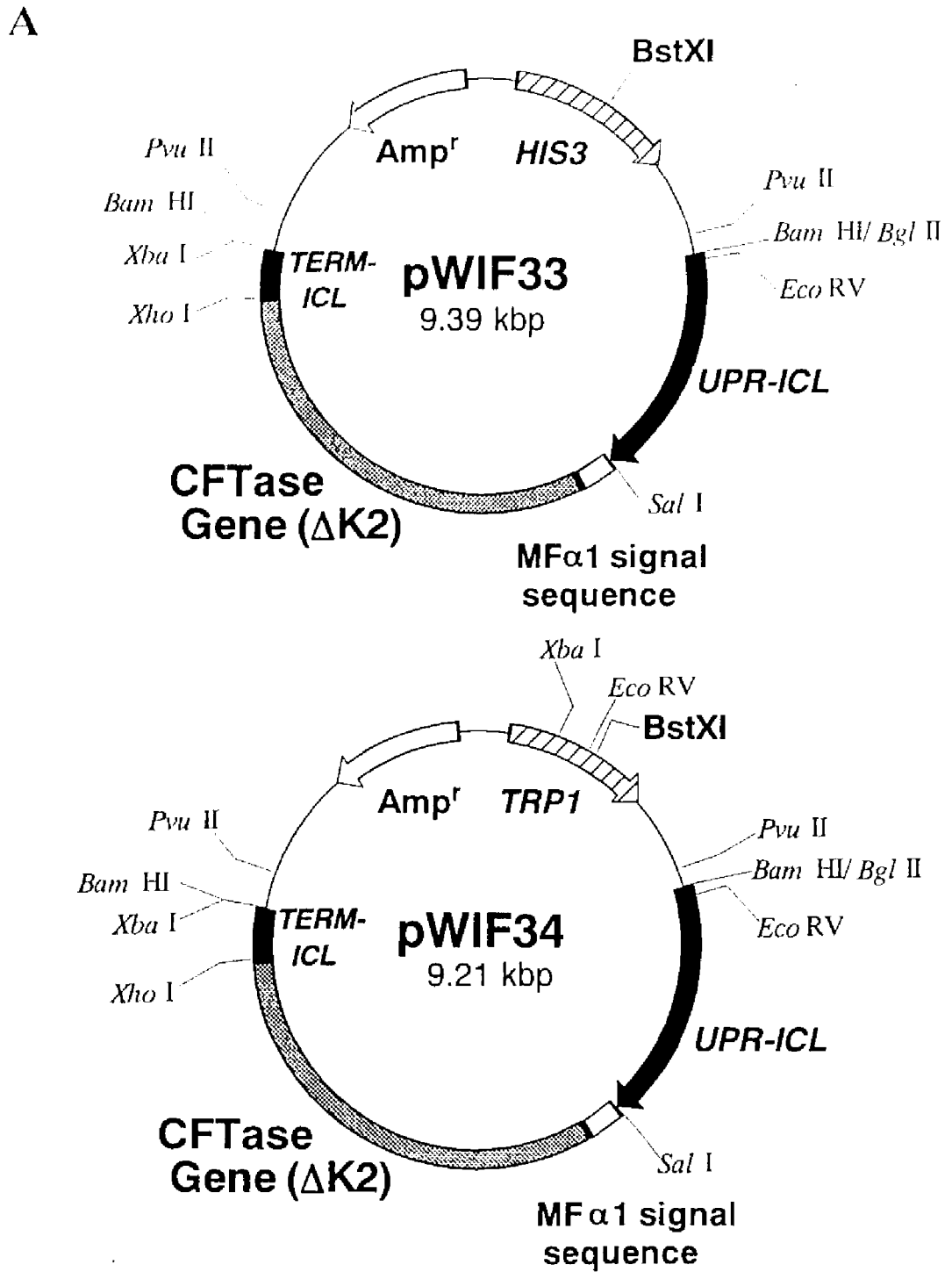


Fig. 4. Structure of pWIF33 and pWIF34 (A), and Southern blot analysis of genomic DNA of strains W303-1A and CF/HW2A (B). (A) Abbreviations of genes were the same as Fig. 1B. (B) *Lanes 1 and 2*, *HIS3* as a probe; *Lanes 3 and 4*, *TRP1* as a probe. *Lanes 1 and 3*, W303-1A genomic DNA digested by *Bst*XI; *Lanes 2 and 4*, CF/HW2A genomic DNA digested by *Bst*XI. *Arrow* indicates intregrated exogenous DNA (9.21 kbp for *HIS3* locus and 9.39 kbp for *TRP1* locus) containing ΔK2 fragment of CFTase gene as well as *HIS3* or *TRP1*.

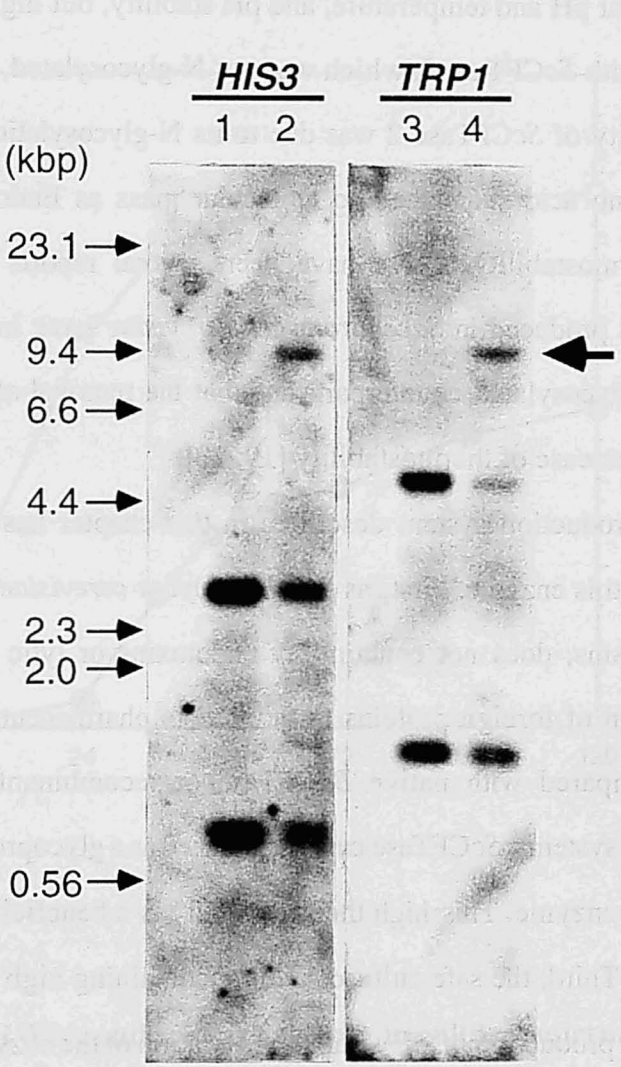


Fig. 4. (Continued).

DISCUSSION

In this chapter, the author reported heterologous expression of a truncated fragment of the CFTase gene from *Bacillus circulans* MCI-2554 in *Saccharomyces cerevisiae*, purification of *Sc* CFTase from culture supernatant, and its characterization. All three *Sc*CFTase molecules showed higher specific activities than that of *Bc*CFTase. The main product, *Sc* CFTase 2, showed similar optimal pH and temperature, and pH stability, but higher thermostability than others. Comparison with *Sc*CFTase 3, which was not N-glycosylated, strongly supported that the high thermostability of *Sc*CFTase 2 was due to its N-glycosylation. *Sc* CFTase 3 had the same N-terminal amino acid sequence and molecular mass as EndoH-treated *Sc*CFTase 2, but showed low thermostability. There have been several reports indicating that the N-glycosylated enzymes produced in *Saccharomyces cerevisiae* have increased thermostability compared to their unglycosylated counterparts and that the removal of N-linked carbohydrate chain(s) results in a decrease of thermostability [19, 20].

The *Sc*CFTase production system described in this chapter has several advantages for further application of this enzyme. First, as *Saccharomyces cerevisiae*, which has the 'GRAS' status of microorganisms, does not contain any endotoxins or lytic viruses, it is a suitable host for the production of foreign proteins to be used in pharmaceutical or food production [9, 10]. Second, compared with native *Bc* CFTase or recombinant CFTase produced by prokaryote expression systems, *Sc*CFTase can be produced as a glycoprotein, making *Sc*CFTase a highly thermostable enzyme. This high thermostability is a beneficial property for enzymes used in industry [21]. Third, the safe culture medium containing high levels of *Sc*CFTase can directly be used for the production of CF without purification of the enzyme, because *Sc* CFTase, like *Bc*CFTase, was efficiently secreted into the culture medium. Moreover, fourth, *Saccharomyces cerevisiae* secretes invertase into the culture medium, which helps to hydrolyze sucrose generated from inulin during the enzyme reaction. This process prevents an increase in the concentration of sucrose in the reaction medium, which would otherwise result in high viscosity of the solution and create problems in the purification of CF.

By shake-flask cultivation, high production (8.40 mg/L) of the *Sc* CFTase protein was achieved by the CF/HW2A strain with two copies of CFTase gene integrated in its chromosomes. Chromosomal integration offers a stable alternative to episomal maintenance of foreign DNA. Integrated DNA is mitotically quite stable; typical rate of vector loss being less than 1% in

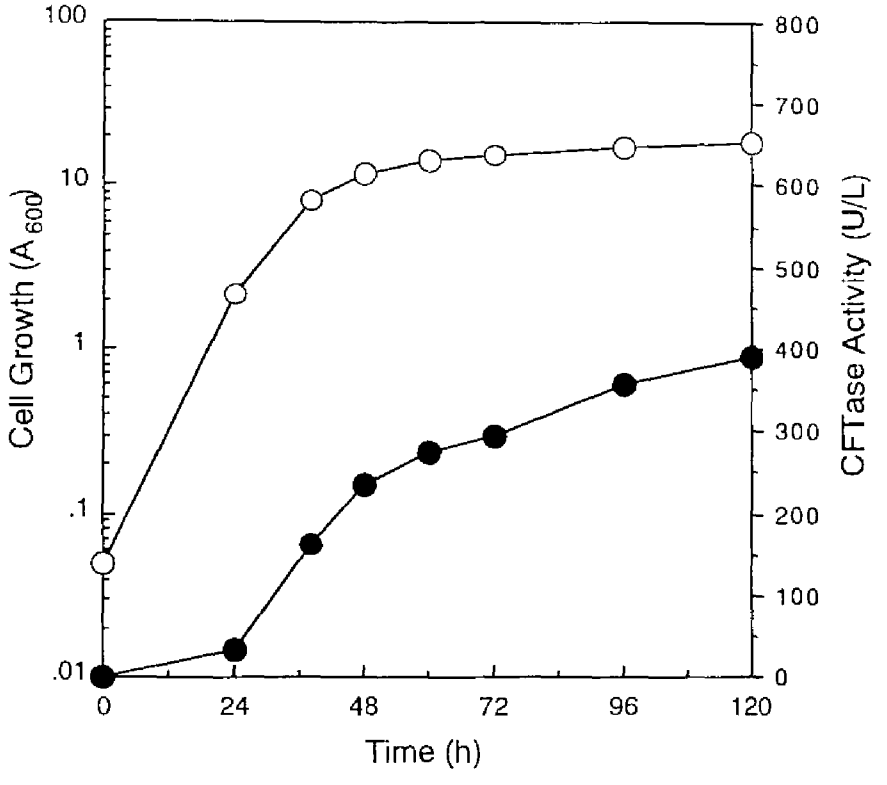


Fig. 5. Growth of CF/HW2A and CFTase activity in culture supernatant. ○, Cell growth (absorbance at 600 nm); ●, CFTase activity in culture supernatant (units per liter culture).

yeast [22]. The exogenous DNA introduced into CF/HW2A chromosome was mitotically stable during repeated cultivation in rich medium. Therefore, increase in the copy number of the CFTase gene will contribute to raise CFTase productivity. For example, more than 20 copies of integration were achieved using δ-sequence of the yeast retorotransposon *Ty* [23]. Moreover, when *Saccharomyces cerevisiae* is cultivated under optimized conditions, cell densities of 50 to 100 mg dry cells per ml culture can be obtained [24]. Therefore, by optimizing fermentation conditions, this system will be promising to provide safe and stable production of CFTase, which can be applied directly to produce CF as it is in the culture medium.

SUMMARY

A truncated fragment of the cycloinulo-oligosaccharide fructanotransferase (CFTase) gene of *Bacillus circulans* MCI-2554 was fused to the prepro secretion sequence of the α-factor and expressed in *Saccharomyces cerevisiae* under the control of 5'-upstream region of the isocitrate lyase gene of *Candida tropicalis* (*UPR-ICL*). Efficiently secreted recombinant CFTase protein (*Sc*CFTase) was purified. *Sc*CFTase consisted of three protein molecules each of which had CFTase activity (*Sc*CFTase 1 [116 kDa], *Sc*CFTase 2 [117 kDa], *Sc*CFTase 3 [116 kDa]). *Sc*CFTase 2 was the major product of the expression system employed and was shown to be N-glycosylated by endoglycosidase H treatment. *Sc*CFTase 1 was also N-glycosylated but had a short truncation at its N-terminus, while *Sc*CFTase 3 did not contain an N-glycosylated carbohydrate chain(s). *Sc*CFTase 2 showed an optimum pH, an optimum temperature, and a pH stability similar to those of CFTase purified from *Bacillus circulans* MCI-2554 (*Bc*CFTase), but exhibited a significant increase in thermostability. While *Bc*CFTase completely lost its activity after 30 min incubation at 70 °C, over 50% of *Sc*CFTase 2 activity was retained even after 30 min at 80 °C. This high thermostability could not be observed with *Sc*CFTase 3, which suggests that the increase in thermostability was due to N-glycosylation of the protein. Next, a strain of *Saccharomyces cerevisiae* was constructed which had two copies of CFTase gene integrated into its chromosomes (CF/HW2A). The integrated exogenous DNA sequence was mitotically stable in the CF/HW2A strain after repeated cultivation in rich medium. Production of *Sc*CFTase by the CF/HW2A strain reached 391 U per liter of culture at 120 h, which corresponded to 8.40 mg of protein per liter, by shake-flask cultivation.

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Part II Analysis of the transcriptional regulation mechanism of *UPR-ICL*

Chapter 1. A novel regulatory factor, Fil1p, involved in glucose repression/derepression in *Saccharomyces cerevisiae*

INTRODUCTION

Most yeast cells can utilize nonfermentable compounds (such as glycerol, lactate, ethanol, or acetate) as a sole carbon source other than fermentable sugars. However, the cells also require hexose phosphates for growth, which is necessary for cell wall and nucleotide biosynthesis. To synthesize such compounds from nonfermentable carbon sources in the cells, enzymes of the glyoxylate cycle and of the gluconeogenic pathway are induced.

Isocitrate lyase is a key enzyme of glyoxylate cycle. In the yeast *Saccharomyces cerevisiae*, the gene coding for isocitrate lyase (*ICL1*) is strictly regulated by carbon sources [1,2]. Its transcription is repressed by glucose and derepressed by nonfermentable carbon sources. Analysis of promoter elements necessary for the derepression of *ICL1* revealed that there exists a upstream activation sequence (UAS) [1-3]. This UAS is called the carbon source-responsive element (CSRE) [3], and its consensus sequence was suggested (CCRTYCRTCCG; where R=A or G, Y=C or T) [4,5]. Similar sequence elements were also found upstream of the fructose 1,6-bisphosphatase gene *FBP1* [6-8], the phosphoenolpyruvate carboxykinase gene *PCK1* [9], the malate synthase gene *MLS1* [4], and the acetyl-coenzyme A synthetase gene *ACS1* [5,10]. Thus, these enzymes would share a common regulatory mechanism for the derepression of the structural genes.

Various *trans*-regulatory factors are identified to be involved in the CSRE-dependent gene regulation. Snf1p (Cat1p) is a serine/threonine protein kinase interacting with Snf4p (Cat3p) to constitute a protein complex with full catalytic activity [11]. This kinase activity of the complex plays a key role for the derepression of various genes. In *snf1* null-mutant cells, derepression of *ICL1*, *MLS1*, and *FBP1* and CSRE-dependent gene regulation do not occur [1,3,4,8]. The *CAT8* and *CAT5* have been also identified as necessary for derepression of *ICL1*, *FBP1*, and *PCK1* and known to be under the control of the *SNF1* [12-14]. Deletion of

CAT8 or *CAT5* has no effects on the expression of *SUC2* (encoding invertase) and *MAL* genes (encoding proteins necessary for maltose utilization), all of which are under the control of *SNF1*. This indicates the existence of distinct pathways regulating *ICL1*, *FBP1*, and *PCK1* genes in the downstream of *SNF1*. It has been shown that the expression of fructose 1,6-bisphosphatase and phospho*enol*pyruvate carboxykinase has a correlation with the phosphorylation of Cat8p [15]. Recently, a 27-kDa protein was identified which can bind to UAS of *ICL1* [16]. The *MIG1/CAT4* encoding a zinc-finger DNA binding protein is necessary for repression of gene expression in cells grown on glucose and has been shown to be involved in the regulation of the *SUC2* and *GAL* genes [17,18]. It is also responsible for the repression of *CAT8* gene expression in glucose-grown cells [13,15].

The gene encoding isocitrate lyase from an *n*-alkane-assimilating yeast *Candida tropicalis* was already cloned [19]. When the gene together with its 5'-upstream region (*UPR-ICL*) was introduced into *Saccharomyces cerevisiae*, its expression was also regulated in response to carbon source as in *Candida tropicalis*. Cells grown on acetate showed a high increase in gene expression compared to cells grown on glucose, suggesting the existence of *cis*-acting elements in *UPR-ICL*, which support strong and regulative gene expression in *Saccharomyces cerevisiae* [20, 21]. The deletion analysis of *UPR-ICL* indicated that two regions (region A1 and region A2) were able to independently activate gene expression in acetate-grown cells. Region A2 contained a consensus sequence of CSRE, and was regulated by *SNF1* and *CAT8*; whereas region A1 which contained no consensus sequence of CSRE, was under the control of *SNF1* and *MIG1* but not *CAT8* [22].

In this chapter, the author describes the cloning and characterization of *FIL1*, which is involved in derepression of the *UPR-ICL*-mediated gene expression as well as of *ICL1* and *FBP1* in *Saccharomyces cerevisiae*. Fil1p, having a mitochondrial targeting sequence at its N-terminus, showed sequence similarity to ribosome recycling factors (RRFs) of prokaryotes, which are presently not found in eukaryotic cells. The author also found that derepression of *ICL1* and *FBP1* as well as *UPR-ICL*-mediated gene expression did not occur in ρ° strain or ρ^{+} cells treated with chloramphenicol or antimycin A, as was the case in the Δ*fil1* strain.

MATERIALS AND METHODS

Yeast strains and media

Saccharomyces cerevisiae strain MT8-1 (*MATa, ade, his3, leu2, trp1, ura3*) [23] was used as wild-type strain (WT) in this study. ρ° Strain was obtained from MT8-1 by repeated cultivation in the YPD medium (1% yeast extract, 2% peptone, 2% glucose) containing 20 mg/ml ethidium bromide [24].

Cells were precultivated on YPD medium, then grown on selective medium (0.67% yeast nitrogen base without amino acids, 0.002% adenine sulfate, 0.02% uracil, 0.02% L-histidine-HCl, 0.002% L-tryptophan and 0.003% L-leucine; in the case of plasmid-transformed strain, corresponding supplements were omitted) containing 2% or 4% glucose (SD), 2% raffinose, or 1% sodium acetate (SA) as a carbon source. Chloramphenicol (CA; 5 g/L) or antimycin A (AA; 200 μg/L) was added to the medium as necessary. For investigation of gene expression in cells cultivated in the medium containing acetate, cells were inoculated at A_{600}=1.0.

Mutagenesis and isolation of mutants

Yeast strain MT8-1 transformed with pMIZ21 (described in Part I, Chapter 1), a multicopy plasmid containing *UPR-ICL-lacZ* fusion gene, was treated with ultraviolet light, and incubated on SD (2% glucose) agar medium at 30 °C for 48 h. Colonies were replicated to SA agar medium containing 40 mg/L 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (SAX medium) and white colonies were chosen and again spotted on SAX agar medium. White colonies were used for measuring β-galactosidase activity.

Cloning and disruption of *FIL1*

The pRS414-based yeast genomic library was constructed as follows; genomic DNA of *Saccharomyces cerevisiae* MT8-1 was digested by *Sau*3AI and fragments longer than 2-kbp were fractionated by a NaCl density gradient. Isolated fragments were inserted into the *Bam*HI site of the single-copy plasmid pRS414.

FIL1 disruption vector (pTU109) was constructed as follows; a *Kpn*I-*Sac*I fragment (1.8-kbp) from pTU105 was first subcloned into pBluescriptII KS' and then a *Hin*dIII-*Hin*dIII fragment (0.7-kbp) was substituted into *HIS3*. pTU109 was linearized by *Hin*cII-*Eco*RV digestion and introduced into *Saccharomyces cerevisiae* MT8-1.

In order to construct pMV1Z, a 478-bp upstream region of *FIL1* (*UPR-FIL1*) together with a part of open reading frame encoding N-terminal 46 amino acids was amplified with the following primers; 5'-TCATCCATGGCAATTTCATCTTCTTCGAC-3' and the M13 reverse primer. The *FIL1* terminator region (681-bp; *TERM-FIL1*) was amplified with the following primers; 5'-CCCCCCATGGATTACGTCGACAAGCTTCATGACCAATTCC-3' and the M13 (-20) primer. These fragments were cut by *Kpn*I, *Sac*I and *Nco*I, and inserted between *Kpn*I and *Sac*I sites of a multicopy plasmid, pMW1 (described in Part I, Chapter 1). The constructed vector was cut by *Nco*I, and *LacZ* which was excised from pMIZ21 was introduced. pMV2Z was constructed essentially by the same method as pMV1Z except that following primers were used to amplify *UPR-FIL1*; 5'-AAAACCATGGTTTCTTGCTGTTATTTTTCTTGT-CAGGGACT-3' and the M13 reverse primer.

Subcellular fractionation

The subcellular fractionation of yeast cells was carried out essentially by the same method reported Daum *et al.* [25] and Kamada *et al.* [26], except that cells grown on raffinose were used. Cells were harvested at late exponential phase (36h for the wild-type strain (WT), 48h for Δ*fil1*, 60h for ρ°, 84h for the chloramphenicol-treated strain, and 60h for the antimycin A-treated strain). After cell lysis with Zymolyase 20T, protoplasts were homogenizd with a teflon homogenizer, and homogenate was centrifuged at 3,000 *g* for 10 min. The postnuclear supernatant obtained was again centrifuged at 20,000 *g* for 15 min to separate a paticulate fraction (P_2, mitochondria) and a supernatant (S_2, cytosol and microsomes). P_2 fraction in this study was almost exclusively occupied by mitochondria.

Proteinase K treatment

The P_2 fraction was treated at 30 °C for 60 min with 2mg/ml proteinase K in the presence or absence of 1% (v/v) Triton X-100. The reaction was terminated by the addition of 1mM (final) phenylmethanesulfonyl fluoride (PMSF).

Enzyme assays

β-Galactosidase activity was assayed as described in Part I, Chapter 1. Isocitrate lyase activity [27], cytochrome *c* oxidase activity [28], NAD^+-linked isocitrate dehydrogenase activity [29], and glucose 6-phosphate dehydrogenase activity [29] were assayed by the method reported previously. All data in tables of this paper are the mean values of two independent

experiments.

Northern blot analysis

The cells were precultured on YPD, shifted to SD (4% glucose) or SA (1% sodium acetate) and incubated for 16 h (initial cell density for SD, A_{600}=0.1; for SA, A_{600}=1.0). Total RNA isolated was separated by 1.5% agarose / 3-morpholinopropanesulfonic acid (MOPS) / formaldehyde gel and detection of the respective mRNA was carried out using the DIG system (Roche Diagnostics, Basel, Switzerland). *ICL1*, *FBP1* and *ZWF1* coding regions used as probes were amplified from *Saccharomyces cerevisiae* genomic DNA by PCR.

Nucleotide sequence accession number

The nucleotide sequence of the *FIL1* gene from *Saccharomyces cerevisiae* was submitted to the GenBank, EMBL, and DDBJ data banks under accession no. AB016033.

RESULTS

Isolation of mutants harboring defects in the derepression of *UPR-ICL*-mediated gene expression and identification of a novel gene *FIL1*.

In order to isolate genes involved in the regulation of *UPR-ICL*-mediated gene expression in *Saccharomyces cerevisiae*, the wild-type strain MT8-1 was first transformed with pMIZ21, a multicopy plasmid which harbored a *UPR-ICL-lacZ* fusion gene together with 2-micron sequence (described in Part I, Chapter 1). Transformed cells formed blue colonies on X-gal-containing agar medium when acetate was used as the sole carbon source (SAX medium). After mutagenesis by ultraviolet light treatment, white colonies on SAX medium were chosen as candidates for mutant strains showing deficiency in *UPR-ICL*-mediated gene expression. Among the mutant candidates, the author chose to conduct further studies with the strain named 1-56-43. The strain 1-56-43 could not derepress the *UPR-ICL-lacZ* fusion gene, and additionally, had a deficiency in derepression of the endogenous isocitrate lyase gene (*ICL1*) (Table 1). Mating with the wild-type α strain followed by tetrad analysis showed that the mutation in the strain 1-56-43 was recessive and due to a single-gene defect. Furthermore, mating with *snf1* and *cat8* null-mutant strains showed that the single-gene mutation was not allelic to *snf1* or *cat8*. Therefore, the author designated the mutation as *fil1* (Factor for Isocitrate Lyase expression).

Table 1. Specific activities of β-galactosidase and isocitrate lyase in cell-free extracts. Cells were harvested after 40h. [a]G, glucose; [b]R, raffinose; [c]A, acetate; [d]complement, strain 1-56-43 harboring pTU105; [e]n.d., not determined.

Strain	β-Galactosidase (mmol min^{-1} mg^{-1})			Isocitrate lyase (nmol min^{-1} mg^{-1})		
	G[a]	R[b]	A[c]	G[a]	R[b]	A[c]
Wild Type	0.05	1.7	13	3.3	5.7	110
1-56-43	0.04	n.d.[e]	0.06	0.0	n.d.[e]	0.0
Complement[d]	5.5	n.d.[e]	14	47	n.d.[e]	84
Δ*fil1*	0.04	0.02	0.01	2.0	1.4	0.0

The mutant strain was transformed with a pRS414-based *Saccharomyces cerevisiae* genomic DNA library in order to identify a gene that complement the *fil1* mutation. One blue colony on SAX medium was obtained, and the isolated plasmid (pTU105) carried a 6.4-kbp genomic DNA fragment. When the mutant cells harboring pTU105 were incubated in medium containing acetate, enzymatic activities of both β-galactosidase and isocitrate lyase were restored when compared to the wild-type strain (Table 1). Increases in enzyme activities could also be detected in cells grown on glucose, which may be attributed to the other region inserted with *FIL1* since they were not observed by the vector alone. Deletion analysis of this DNA fragment revealed that pTU108, which harbored a 1.8-kbp *Kpn*I-*Sac*I fragment, was sufficient to complement the *fil1* mutant phenotype (Fig. 1A).

Fil1p shows similarity to ribosome recycling factors of prokaryotes.

The nucleotide sequence of the *Kpn*I-*Sac*I fragment was deteremined, and found to be identical to a region between *PUT2* and *SRB2* on chromosome VIII (GenBank accession number U00062) [30]. There was one open reading frame (identical to YHR038w) which encodes a 230-amino-acid and lysine-rich (15.2%) protein (Fig.1B). From the mutant strain 1-56-43, the *FIL1* gene was amplified by PCR and sequence analysis was performed for three independently amplified fragments. In all fragments, a nonsense mutation was incorporated

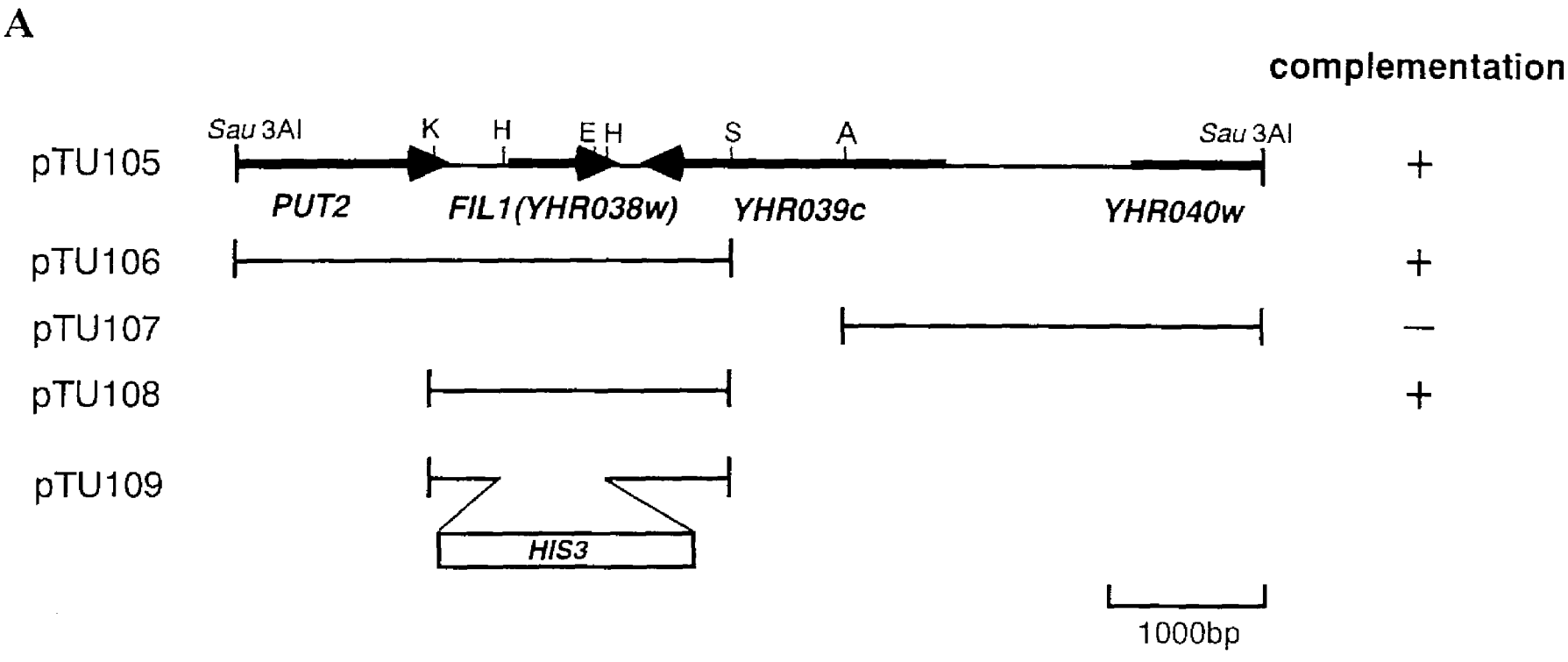

Fig. 1. Isolation of the *FIL1* gene encoding a protein with similarity to prokaryotic ribosome recycling factors. (A) Restriction map of the genomic DNA fragment which complements *fil1*. Restriction sites: A, *Apa*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; S, *Sac*I. Construction strategy for the *fil1::HIS3* disruption vector (pTU109) is shown in the bottom. (B) Nucleotide sequence of *FIL1* and deduced amino acid sequence. The cleavage-site motif (R-X-F-S) of mitochondrial targeting peptide is boxed. (C) Amino acid alignment of Fil1p and RRFs of prokaryotes [*Chlamydia trachomatis* (GenBank accession number, U60196), *Mycoplasma genitalium* (U39730), *Mycoplasma pneumoniae* (AE000020), *Escherichia coli* (J05113), *Haemophilus influenzae* (U32763), *Synechocystis sp.* (D90915)]. The shaded areas indicate exact matchs or conservative amino acid differences. Asterisks indicate the amino acid residues with higher conservation in all proteins.

B

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(-478)
  GGTACCGACGATAAGGCTGGTGGTCCAAACATTTTAAGCAGATTTGTCAGTATTAGAA
ACACAAAGGAGAACTTCTACGAGTTGACTGATTTCAAATATCCATCGAATTATGAATAAA
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 M  I  L  T  T  A  R  L  N  C  R  P  V  T  V  P  R  L  F  N

CGTTCTTTTAGTCAATCTTTCATAATTTTGAAGAAAAAAAGTTCTACCCCTACTGAGAAA 120
[R  S  F  S] Q  S  F  I  I  L  K  K  K  S  S  T  P  T  E  K

GTCGAAGAAGATGAAATTGACGTGAATGAACTGCTGAAAAAGGCAGAAACTCAATTCAAA 180
 V  E  E  D  E  I  D  V  N  E  L  L  K  K  A  E  T  Q  F  K

AAAACTTTAGAAATTCAAAAACAGAAAATGAATGAGATAAAACAGGGAAATTTTAATCCT 240
 K  T  L  E  I  Q  K  Q  K  M  N  E  I  K  Q  G  N  F  N  P

AAGGTATTCAATAGTTTAGTGTTCAAAAATAACAGAAAGTTTACAGATATTGCTACCACA 300
 K  V  F  N  S  L  V  F  K  N  N  R  K  F  T  D  I  A  T  T

TCCTTGAAAGGTAAAAATGCACTTTTAATAACAGTTTTCGACCCCAAAGATGTGAAAACT 360
 S  L  K  G  K  N  A  L  L  I  T  V  F  D  P  K  D  V  K  T

GTGATCAGTGGGGTGCTTGCTGCGAACCTGAATTTAACTCCTGAAAGGGTCCCAAATAAC 420
 V  I  S  G  V  L  A  A  N  L  N  L  T  P  E  R  V  P  N  N

GATTTGCAATTGAAAGTTTCGTTACCACCACCAACTACAGAATCCCGGTTAAAAGTAGCT 480
 D  L  Q  L  K  V  S  L  P  P  P  T  T  E  S  R  L  K  V  A

AAAGACTTAAAGAGAGTATTTGAAGAATATAAGCAGTCATCGCTAAAAGACTCATTAGGA 540
 K  D  L  K  R  V  F  E  E  Y  K  Q  S  S  L  K  D  S  L  G

ACTATCAGAGGCAGTATTCTAAAGGAATTCAAAAGTTTCAAAAAGGATGATGCCGTTCGA 600
 T  I  R  G  S  I  L  K  E  F  K  S  F  K  K  D  D  A  V  R

AAAGCTGAGAGGGATTTGGAAAAACTGCATAAGGATTACGTGAACAAGCTTCATGACCAA 660
 K  A  E  R  D  L  E  K  L  H  K  D  Y  V  N  K  L  H  D  Q  .

TTCCAGAAAGTTGAAAAAAGCATTGTAAAATGAGGAATTTCGTAACGTGTATAGAATCAA 720
 F  Q  K  V  E  K  S  I  V  K  *  (230 aa)

AAAATCATAAATCTTGTACATAAAAAGTTCATTTTATATAATAATAAATACCAAATTATT
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TTGCATTCCTTGATATCCGCACCAAACACAGAACCACCTAGACCAAATGGCGCAGAGTTG
GCTAGTTGTACACAATGGTCAGTATTCTTAGCTTTCATCATGACTAAAATTGGGCCAAAC
ACTTCGTTTTGTGCTATTTTCATTTCTGGAGTGACATCCACCAAAAGAGTTGGTTGGAAA
TAATGACCTTGTGGATACTTTGGATGTTTGAAGCGGGAACCACCTTGAAGTAAACGAGCT
C (1381)
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Fig. 1. (Continued).

C

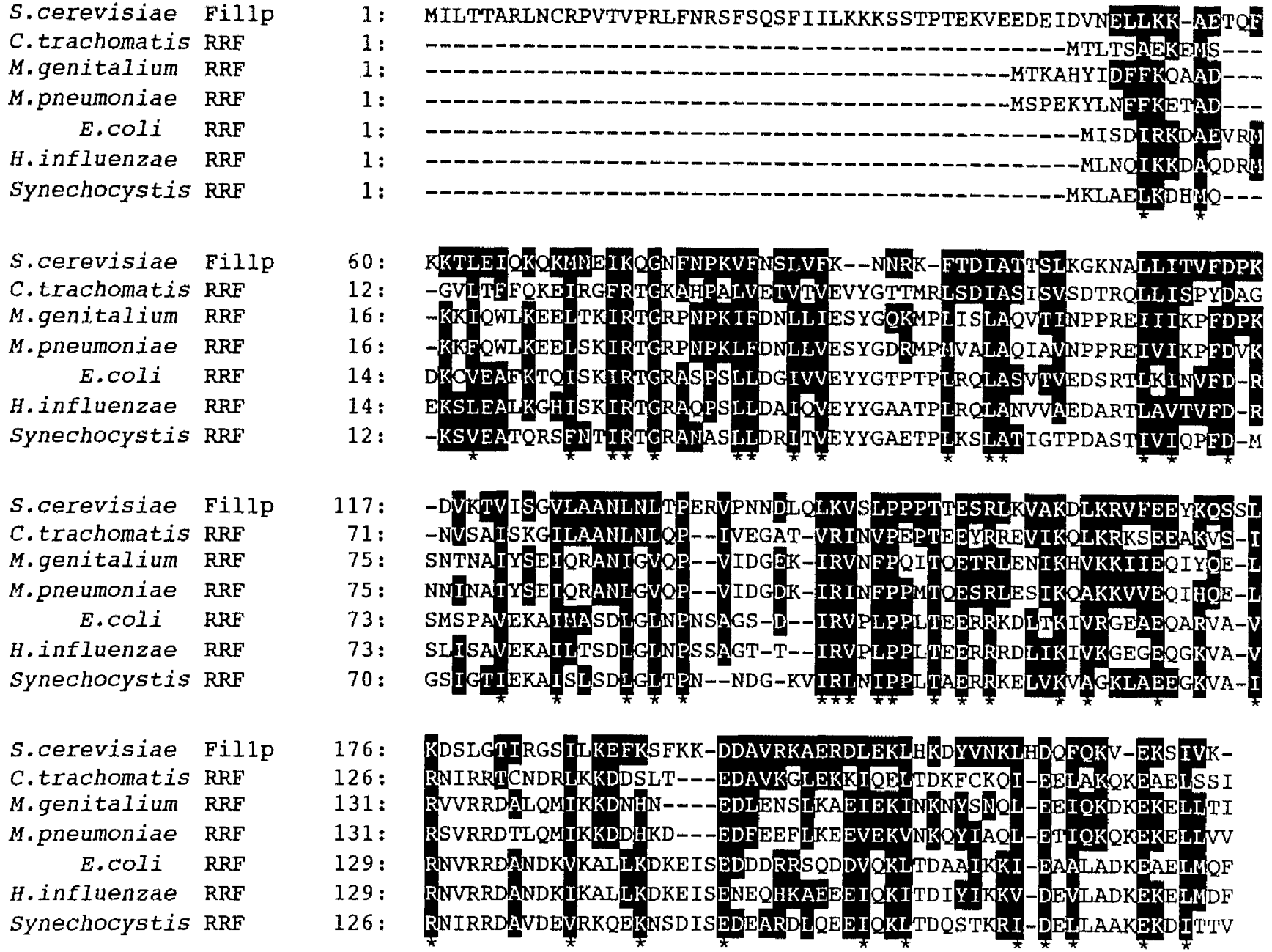

Fig.1. (Continued).

by T → A transition at the position 188 (at Leu63). Data base analysis with the amino acid sequence of Fil1p showed that it was similar to ribosome recycling (or releasing) factors (RRFs) of prokaryotes (Fig.1C). Fil1p had 51% similarity to RRF of *Mycoplasma genitalium*, 46% to that of *Chlamydia trachomatis* and 44% to that of *Escherichia coli*. RRF of *Escherichia coli* is an essential factor for growth, and functions to release ribosomes from mRNA following the termination of protein synthesis at the stop codon [31], while the cooperative function with the other factor(s) has been recently reported [32]. Although there has been a report of a carrot protein called NLP (nuclear located protein D2, GenBank accession number X72384) [31], which does not contain an N-terminal extension but shows 65% sequence similarity to RRF of *Escherichia coli*, an RRF in eukaryotic cells has yet not been identified with genetic and/or biochemical evidence.

Using pTU109 (Fig.1A), *FIL1* deletion mutant strain (Δ*fil1* strain) was constructed. Successful disruption of *FIL1* was verified by both PCR and Southern blot analysis. The Δ*fil1* strain could grow on glucose and raffinose, but not on non-fermentable carbon sources such as acetate, glycerol, lactate or oleate. β-Galactosidase and endogenous isocitrate lyase activities were then measured using the Δ*fil1* strain transformed with pMIZ21. Derepression of both enzymes could not be observed (Table 1), confirming that *FIL1* is necessary for both the regulation of *UPR-ICL*-mediated gene expression and *Saccharomyces cerevisiae ICL1* gene expression.

Fil1p contains a targeting sequence into mitochondria.

Alignment of Fil1p with RRFs of prokaryotes revealed that Fil1p has an extension at its N-terminus (Fig. 1C). Within this extension, there is the sequence '-Arg-X-Phe-Ser-' (Fig. 1B), which is a consensus sequence for the cleavage site often observed for mitochondrial targeting peptides [33]. These facts suggest that the N-terminal region of Fil1p might function as a transport signal into mitochondria. To test this hypothesis, pMV1Z and pMV2Z were constructed which express β-galactosidase protein fused with or without, respectively, the N-terminal 46 amino acids of Fil1p (Fig.2). After transforming the fusion genes into wild-type cells, The P_2 fraction was isolated from raffinose-grown cells and β-galactosidase activity was measured. The P_2 fraction isolated from raffinose-grown cells was almost exclusively occupied by mitochondria. The activity of cytochrome *c* oxidase was used as a marker protein

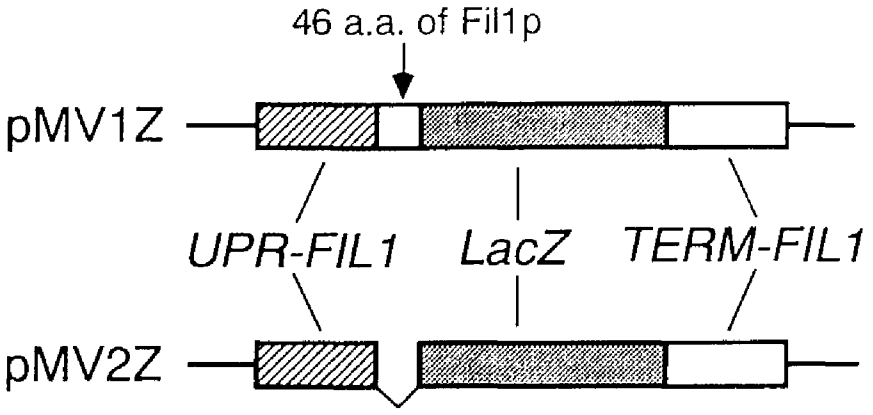

Fig. 2. Structure of plasmids pMV1Z and pMV2Z. *UPR-FIL1*, 5'-upstream region of *FIL1*; *TERM-FIL1*, terminator region of *FIL1*. Between *UPR-FIL1* and *LacZ*, pMV1Z contains a nucleotide sequence encoding the N-terminal 46-amino acid residue of Fil1p.

for mitochondria. As for pMV1Z-transformed strain, a significant level of β-galactosidase activity (21%) was detected in P_2 fraction, while no β-galactosidase activity could be observed in the P_2 fraction isolated from pMV2Z-transformed strain (Table 2). The P_2 fraction isolated from pMV1Z-transformed cells was further treated with proteinase K and detergent (Table 3). Most of β-galactosidase activity (92 %) could be recovered even after proteinase K treatment. When Triton X-100 was added along with proteinase K, β-galactosidase activity completely diminished, indicating that β-galactosidase was localized within the mitochondria. These results indicate that the N-terminal 46 amino acids of Fil1p can function as a mitochondrial-target peptide and strongly suggest that Fil1p is localized and functions in mitochondria. The author have also confirmed that a deletion fragment of Fil1p, which lacks only the N-terminal 46 amino acids, could not complement the Δ*fil1* strain .

Fil1p is involved in protein synthesis in mitochondria.

Many previous studies have shown that the mechanisms and protein factors involved in transcription and translation in mitochondria resemble those found in prokaryotic cells (reviewed in [34, 35]). As Fil1p contains a mitochondrial targeting sequence and shows high sequence similarity to prokaryotic RRFs, the author supposed that Fil1p functions as a mitochondrial

Table 2. Total activities of β-galactosidase and cytochrome *c* oxidase in S_2 and P_2 fractions of pMV1Z- and pMV2Z-transformed wild type strains. Both cells were grown on raffinose as a carbon source and subjected to subcellular fractionation as described in Materials and Methods.

	β-Galactosidase		Cytochrome *c* Oxidase	
Plasmid : Fraction	(nmol min^{-1})		(μmol min^{-1})	
pMV1Z : S_2	310	(79%)	8.6	(29%)
: P_2	81	(21%)	21	(71%)
pMV2Z : S_2	140	(100%)	1.7	(8%)
: P_2	0.0	(0%)	20	(92%)

Table 3. Proteinase K treatment of P_2 fraction isolated from pMV1Z-transformed strain with or without Triton X-100. The value 100% refers to the activity of P_2 fraction without proteinase K or Triton X-100 (None).

Treatment	β-Galactosidase Activity (%)
None	100
with Proteinase K	92
with Triton X-100	100
with Proteinase K and Triton X-100	0

RRF involved in protein synthesis in mitochondria. Therefore, the subcellular fractionation was performed on raffinose-grown Δ*fil1* cells, and proteins of the cytosol and microsome fraction (S_2 fraction) and mitochondrial fraction (P_2 fraction) were compared with the corresponding fractions from wild-type cells. As a result of SDS-PAGE analysis, some proteins observed in the P_2 fraction of the wild-type strain (arrowhead) could not be observed in the Δ*fil1* strain (Fig. 3, lanes 1 and 2). On the other hand, few different bands could be observed in protein constituents of S_2 fraction between these strains (Fig.3, lanes 6 and 7).

Further investigation was carried out to determine whether or not the proteins whose levels decreased in the Δ*fil1* strain were due to defects in the protein synthesis in the mitochondria. The author constructed a ρ^0 strain from the wild-type MT8-1 which would lack the mitochondrial DNA. Wild-type cells were also treated with chloramphenicol, which specifically inhibits protein synthesis in mitochondria. These cells, along with Δ*fil1* cells, were grown on raffinose and protein constituents in the P_2 and S_2 fractions were compared. By SDS-PAGE analysis of the P_2 fraction, Δ*fil1*, chloramphenicol-treated, and ρ^0 cells all showed similar protein constituents (Fig.3, lanes 2-4). The proteins whose levels decreased in Δ*fil1* cells compared to wild-type cells were also found to be at low levels in chloramphenicol-treated cells and ρ^0 cells. In contrast, no significant differences in protein constituents of S_2 fraction could be found in any of the cells investigated (Fig. 3, lanes 6-9).

Cytochrome *c* oxidase activity in the P_2 fraction was also compared among these cells. Cytochrome *c* oxidase is a multi-subunit complex localized in mitochondrial inner membrane, three of whose subunits are encoded by the mitochondrial genes (*COX1-3*) and synthesized in mitochondria, while the other subunits are encoded by nuclear genes. Cytochrome *c* oxidase activity could not be detected in ρ^0 and chloramphenicol-treated cells, and only trivial activity could be found in Δ*fil1* cells compared with wild-type cells (Table 4), although the addition of antimycin A, which can stoichiometrically associate with ubiquinone-cytochrome reductase and inhibit electron transfer from cytochrome *b* to cytochrome c_1, had no effect on that enzyme activity and on the protein synthesis in mitochondria (as seen in Fig. 3, lane 5). The specific activity of NAD^+-linked isocitrate dehydrogenase, a protein encoded by the nuclear gene and transported post-translationally into mitochondria, was not affected in each cell. These results further support the proposal that Fil1p is necessary for efficient protein synthesis

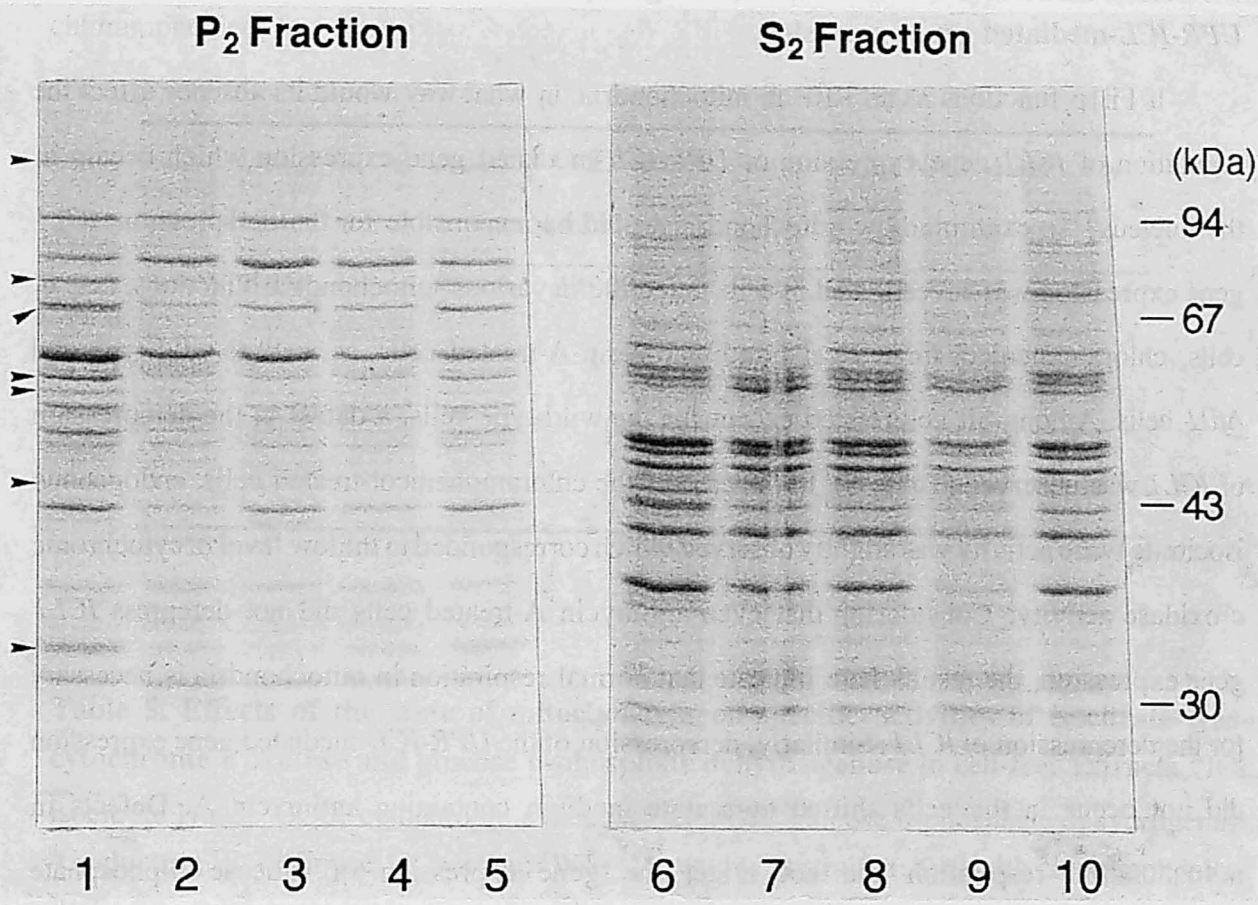

Fig. 3. Difference in protein constituents between wild-type, *Δfil1*, ρ°, and chloramphenicol-treated strains. All cells were grown on raffinose as a carbon source and subject to subcellular fractionation as described in Materials and Methods (each lane, 50 μg protein). *Lanes 1 and 6*, wild-type; *lanes 2 and 7*, *Δfil1*; *lanes 3 and 8*, ρ°; *lanes 4 and 9*, chloramphenicol-treated strain; *lanes 5 and 10*, antimycin-A-treated strain. *Lanes1 to 5*, P_2 fraction (mitochondria); *lane 6 to 10*, S_2 fraction (cytosol and microsomes). Arrowheads represent proteins which can only be observed in the P_2 fraction of the wild-type strain.

in mitochondria and its possible role as an RRF.

Mitochondrial respiration is necessary for the expression of *ICL1* and *FBP1* and the *UPR-ICL*-mediated gene expression.

If Fil1p functions as an RRF in mitochondria, in what way would its absence affect the regulation of *ICL1* gene expression or *UPR-ICL*-mediated gene expression which occurs in the nucleus? To examine how mitochondria would be responsible for the derepression, *ICL1* gene expression was investigated in cells defective in various mitochondrial functions, i.e., ρ° cells, chloramphenicol-treated cells and antimycin-A-treated cells, as well as wild-type and *Δfil1* cells. Among all cells tested except for the wild-type cells, a defect in the derepression of *ICL1* was observed (Table 5). In the case of the chloramphenicol-treated cells, endogenous isocitrate lyase activity was slightly observed which corresponded to the low level of cytochrome *c* oxidase activity. Considering that even antimycin A-treated cells did not derepress *ICL1* gene expression, the results here indicate that normal respiration in mitochondria is necessary for the derepression of *ICL1*. Similarly, derepression of the *UPR-ICL*-mediated gene expression did not occur in the cells shifted to acetate medium containing antimycin A. Defects in mitochondrial respiration did not affect the gene expression of glucose 6-phosphate dehydrogenase, whose expression is not regulated by glucose repression and derepression [36].

RNA was isolated from these five strains and Northern blot analysis was performed. Transcription of *ICL1* is normally derepressed in wild-type strain incubated in the medium containing acetate (Fig.4, lane 6), while this derepression could not be observed in all other strains (Fig.4, lanes 7-10). The same result was obtained for the derepression of *FBP1* gene. In contrast, transcription of *ZWF1* encoding glucose 6-phosphate dehydrogenase remained constitutive in all strains after incubation for 16 h (Fig.4, lanes 1-10), which correlates to the enzyme activity observed in the cells (Table 5). These results indicate that defects in mitochondrial respiration inhibits *ICL1* and *FBP1* gene expression at the transcriptional level.

Table 4. Specific activities of cytochrome *c* oxidase and NAD$^+$-linked isocitrate dehydrogenase in P_2 fraction from raffinose-grown cells. [a]CCO, cytochrome *c* oxidase; [b]NAD$^+$-IDH, NAD$^+$-linked isocitrate dehydrogenase; [c]WT+CA, wild type strain treated with chloramphenicol.

Strain	CCO[a] (nmol min^{-1} mg^{-1})	NAD$^+$-IDH[b] (μmol min^{-1} mg^{-1})
Wild Type	1400	0.16
Δfil1	11	0.29
ρ^0	0	0.40
WT+CA[c]	0	0.21

Table 5. Effects of the state of mitochondria on specific activities of isocitrate lyase, cytochrome *c* oxidase and glucose 6-phosphate dehydrogenase in cell-free extracts. [a]ICL, isocitrate lyase; [b]CCO, cytochrome *c* oxidase; [c]G6PDH, glucose 6-phosphate dehydrogenase; [d]G, glucose; [e]R, raffinose; [f]A, acetate; [g]WT+CA, wild type strain treated with chloramphenicol; [h]WT+AA, wild type strain treated with antimycin A. Cells were harvested after 24 h of incubation in the medium containing each carbon source indicated. As for glucose and raffinose media, cells were inoculated at A_{600}=0.05. As for acetate medium, cells were inoculated at A_{600}=1.0.

Strain	ICL[a] (nmol min^{-1} mg^{-1})			CCO[b] (nmol min^{-1} mg^{-1})			G6PDH[c] (μmol min^{-1} mg^{-1})		
	G[d]	R[e]	A[f]	G[d]	R[e]	A[f]	G[d]	R[e]	A[f]
Wild Type	4.3	7.8	140	88	140	95	0.23	0.37	0.34
Δfil1	4.6	2.5	0	6.1	14	2.1	0.27	0.41	0.61
ρ^0	3.0	2.4	0	0	0	0	0.23	0.50	0.44
WT+CA[g]	4.0	2.3	26	3.2	0	8.5	0.28	0.45	0.38
WT+AA[h]	4.2	3.4	0	65	170	23	0.18	0.34	0.52

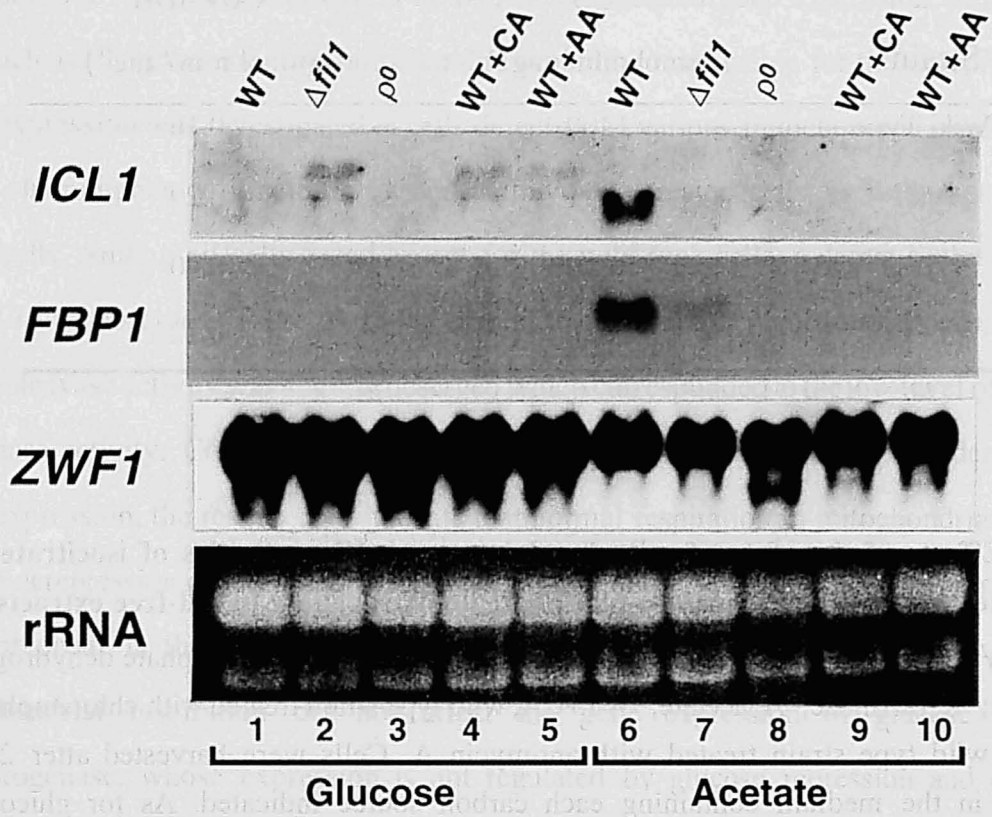

Fig. 4. Derepression of *ICL1* and *FBP1* was affected by the state of mitochondria. Total RNA was isolated from wild-type (*lanes 1 and 6*), Δ*fil1* (*lanes 2 and 7*), ρ° (*lanes 3 and 8*), chloramphenicol-treated (*lanes 4 and 9*), and antimycin A-treated (*lanes 5 and 10*) cells. These cells were precultured on YPD, shifted to SD (4% glucose, *lanes 1-5*) or SA (1% sodium acetate, *lanes 6-10*), and incubated for 16 h. The Northern blots (each lane, 20 μg RNA) were done with probes specific for *ICL1*, *FBP1* or *ZWF1*, each encoding isocitrate lyase, fructose 1,6-bisphosphatase or glucose 6-phosphate dehydrogenase, respectively. A differnce in the size of *ICL1* transcripts was observed between the cells incubated in the medium containing glucose and acetate. To indicate the appropriate conditions in the isolation of total RNAs , the gels after electrophoresis were stained with ethidium bromide.

DISCUSSION

Function of Fil1p and mitochondrial protein synthesis.

In this chapter, the author reported the isolation and identification of *FIL1*, a gene which was found to be necessary for the derepression of *UPR-ICL* in *Saccharomyces cerevisiae* as well as *ICL1* or *FBP1*. Characterization of Fil1p revealed the following facts; (a) high sequence similarity between Fil1p and prokaryotic RRFs, (b) the N-terminal 46 amino acids of Fil1p, which is an extensional region not found in prokaryotic RRFs, functioned as a mitochondrial targeting sequence, (c) lack of Fil1p affected mitochondrial protein constituents, similar to the effects of chloramphenicol and loss of mitochondrial DNA (ρ°), (d) lack of Fil1p led to a drastic decrease in the activity of cytochrome *c* oxidase, a multi-subunit complex which contains subunits synthesized by mitochondrial translation machinery, while the activity of NAD^{+}-linked isocitrate dehydrogenase encoded by the nuclear gene was not affected. From these facts, the author supposes that Fil1p functions in protein synthesis in mitochondria, possibly as a mitochondrial RRF. So far, there is no report of RRF in the mitochondria of eukaryotes, but the mitochondrial presence of an RRF similar in structure to prokaryotic RRFs may be consistent with the similarities in the translational machinery found between the mitochondria and prokaryotic cells (as reviewed in [35]). However, in spite of its similarity, the author still does not have direct biochemical evidence that Fil1p actually functions as an RRF in mitochondria, and the possibility can not be excluded that Fil1p functions in other process of protein synthesis in mitochondria. Further biochemical studies will clarify the exact function of Fil1p, whether Fil1p is a mitochondrial RRF or not.

Effects of the respiratory chain on *ICL1* and *FBP1* expression.

The data provided in this chapter indicate that the normal function of the respiratory chain in mitochondria is necessary for the derepression of *ICL1* and *FBP1*, encoding enzymes of the glyoxylate cycle and gluconeogenic pathway as well as *UPR-ICL*-mediated gene expression in *Saccharomyces cerevisiae*. In this yeast, transcription of *ICL1*, *FBP1* and *PCK1* is regulated, at least partly, by a common machinery which functions through CSRE located on the promoter of each gene. Therefore, the common necessity of mitochondrial respiration on the derepression of these genes is reasonable. It has also been reported that respiration is a

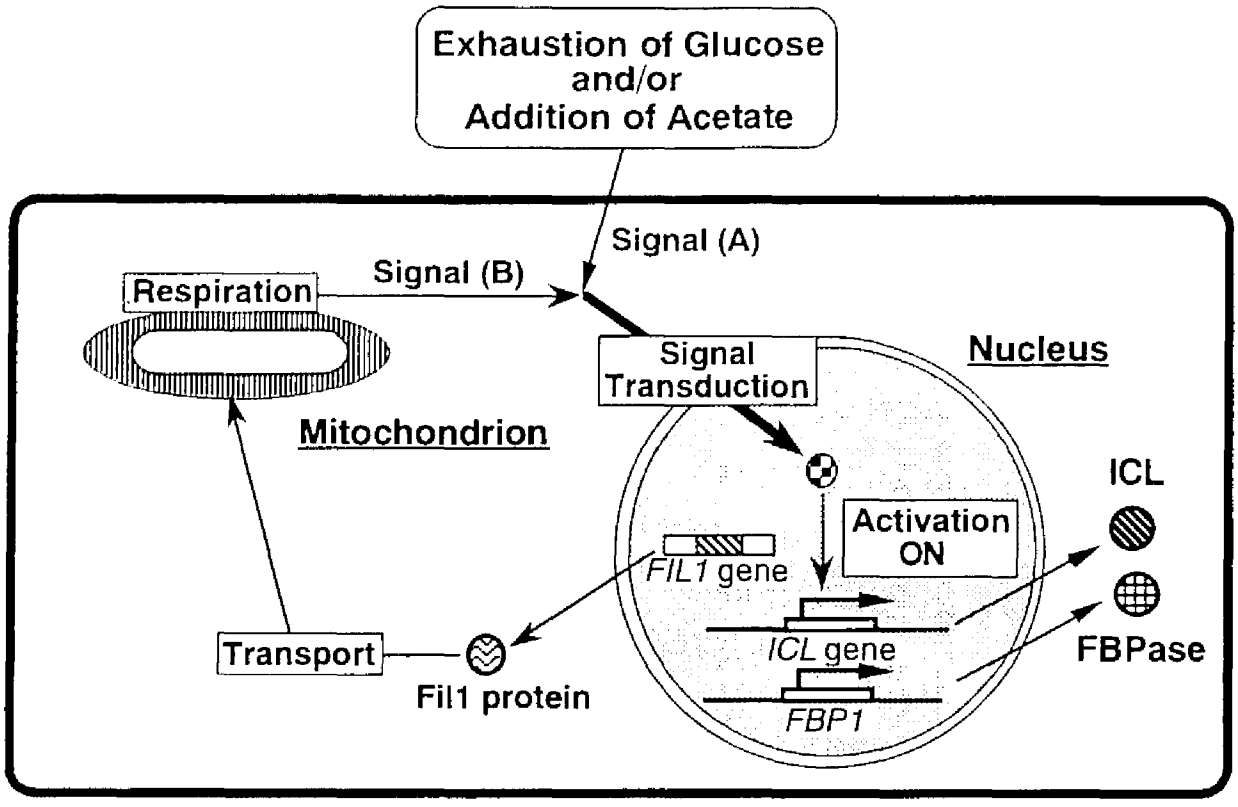

Fig. 5. Organelle communication in regulating glucose repression-derepression. ICL, isocitrate lyase; FBPase, fructose 1,6-bisphosphatase.

necessary factor for the gene expression of the peroxisomal thiolase gene (*POT1*) of the β-oxidation pathway [37]. In contrast, the author has shown that defects in function of the respiratory chain did not affect gene expression of *ZWF1*, encoding glucose 6-phosphate dehydrogenase whose expression is not regulated by a glucose repression/derepression mechanism. It has also been reported that the derepression of the glucose-repressible *SUC2* gene is not affected in the ρ° strain [37].

Recently, *CAT5* was shown to be allelic to *COQ7* encoding a protein necessary for ubiquinone biosynthesis [38]. A *cat5/coq7* null-mutant exhibits a respiration-deficiency phenotype and also derepression of *ICL1*, *FBP1*, and *PCK1* could not be observed in this strain [13, 38, 39]. Comparing these two mutants (*fil1* and *cat5*), it is reasonable to propose that, although both mutations were different, the silmilar mutant phenotype (defects in the derepression of *ICL1*, *FBP1*, and *PCK1*) may be due to the defects in a common intracellular pathway, which is caused by lack of respiration.

It is a striking but an interesting fact that there exists a communication pathway between mitochondria and the nucleus (Fig. 5). In yeast, the enzymes of glyoxylate cycle, gluconeogenesis and β-oxidation pathways, all of which are not located in mitochondria, are induced when cells grow on non-fermentable carbon sources. In such a situation, mitochondrial respiration is an absolutely necessary process for cells to produce energy from acetyl-CoA through the tricarboxylic acid cycle. Therefore, this cell response against the defects of mitochondrial respiration is physiologically relevant. There are several genes of which expression in the nucleus is influenced by the state of mitochondria (reviewed in [35, 40, 41]). *CIT2* encoding the peroxisomal citrate synthase is one such example and its transcription is activated in ρ° strain or ρ^{+} strain treated with antimycin A (retrograde communication) [42, 43], which is in contrast to the case of *ICL1* and *FBP1* where transcription is deactivated in these strains. Retrograde regulation of *CIT2* is controlled through UAS_{retro} located on the promoter, and three factors involved in this regulation (*RTG1-3*) have been isolated at present [43, 44].

It is previously reported that *UPR-ICL* is controlled by two distinct pathways in *Saccharomyces cerevisiae*, one through region A1 which is dependent on *SNF1* but not *CAT8*, and the other through region A2 which contains CSRE consensus sequence and is dependent on *SNF1* and *CAT8* [22]. The fact that *UPR-ICL*-mediated gene expression was completely repressed by the defects in respiration indicates that derepression through region A1 and region A2 (CSRE) are both repressed. This notion was confirmed by the fact that constructs harboring either region A1 or region A2 independently as a UAS could not derepress downstream genes in antimycin A-treated cells. The respiration signal may have effect on factors localized where the regulatory pathways controlling region A1 and region A2 are in common. However the expression of glucose-repressive *SUC2* gene, which is dependent on *SNF1*, was not repressed in the ρ° strain [37]. The effect of mitochondrial respiration on the derepression of *UPR-ICL* (or *ICL1* and *FBP1* as well) may be through a common factor positioned downstream of Snf1p but upstream of Cat8p.

SUMMARY

A mutant was isolated that failed to derepress the 5' upstream region of *Candida tropicalis* isocitrate lyase gene (*UPR-ICL*)-mediated gene expression in acetate medium, and

the gene (*FIL1*) that complemented this mutation was isolated. The *fil1* null-mutant in which *FIL1* is disrupted (*Δfil1* strain) could not grow on acetate or ethanol, and the derepression of the isocitrate lyase encoded by *ICL1* in *Saccharomyces cerevisiae* was also defected. The amino acid sequence of Fil1p (230 amino acids) showed similarity to ribosome recycling factors (RRFs) of prokaryotes. Compared to prokaryotic RRFs, Fil1p had an N-terminal 46 amino acid extension which was shown to be able to function as a mitochondrial-targeting sequence. The subcellular fractionation of the *Δfil1* strain showed that protein constituents of the mitochondrial fraction differed from that of the wild-type strain, but resembled those of chloramphenicol-treated cells or ρ° cells. The specific activity of cytochrome *c* oxidase was severely decreased in *Δfil1*, ρ° and chloramphenicol-treated cells compared to wild-type cells, while enzymatic levels of mitochondrial NAD^{+}-linked isocitrate dehydrogenase, which is encoded by the nuclear DNA, were not affected. These results suggest that Fil1p is necessary for protein synthesis in mitochondria of *Saccharomyces cerevisiae*. Furthermore, the cells treated with antimycin A, along with chloramphenicol-treated, ρ°, and *Δfil1* cells, showed deficiency in derepression of isocitrate lyase. Northern blot analysis showed that this can be ascribed to no increase in transcription of *ICL1* and *FBP1* encoding fructose 1,6-bisphosphatase. The results indicate the presence of a communication pathway between mitochondria and the nucleus which represses expression of genes encoding the key enzymes of the glyoxylate cycle and gluconeogenic pathway when there is a deficiency in the mitochondrial respiratory chain.

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Chapter 2. Genetic evaluation of the function of *SNF1* in *Candida tropicalis*

INTRODUCTION

Glucose repression is the phenomenon that synthesis of enzymes participating in the utilization of carbon sources other than glucose is repressed in the presence of glucose. In yeast *Saccharomyces cerevisiae*, genes encoding enzymes of the alternative sugar utilization (*GAL*, *MEL* or *SUC* genes), the glyoxylate cycle (*ICL1* and *MLS1*), and the gluconeogenesis (*FBP1* and *PCK1*) are under this regulation, and the underlying mechanism has been investigated extensively [1-6]. *SNF1* (also known as *CAT1* or *CCR1*) of *Saccharomyces cerevisiae* (*ScSNF1*) is one of the genes required for derepression of these genes [7-9]. *SNF1* (Sucrose Non-Fermenting) was identified by screening for a *Saccharomyces cerevisiae* mutant unable to grow on sucrose as a sole carbon source [9]. The *snf1* mutant neither grow on galactose, maltose nor nonfermentable carbon sources such as glycerol, ethanol or acetate [7-9]. Furthermore, the *snf1* mutant cell shows pleiotropic phenotype such as loss of glycogen accumulation [10-12], hypersensitivity to heat shock and starvation [10], and loss of sporulation [9].

SNF1 encodes a serine/threonine protein kinase [13] and Snf1 homologs are existing even in plant [14] and mammals [15, 16]. In *Saccharomyces cerevisiae*, Snf1 protein (Snf1p) interacts with other proteins including Snf4 (or Cat3) protein (Snf4p) and the Gal83, Sip1, and Sip2 proteins [17-20]. Snf4p interacts with the regulatory domain of Snf1p, and regulates the activity of Snf1p. The Gal83, Sip1, and Sip2 proteins, which constitute a family of related proteins, are the proposed bridge proteins interacting with both Snf1p and Snf4p [21].

Candida tropicalis is an asporogenic diploid yeast which can utilize *n*-alkanes as a sole carbon source. In assimilating *n*-alkanes, a profound proliferation of peroxisome, a subcellular organelle, is observed [22, 23]. Consistent with peroxisome proliferation, enzymes localized in peroxisome, such as the enzymes of the β-oxidation or the glyoxylate cycle, are also induced. Isocitrate lyase (ICL), a key enzyme of the glyoxylate cycle and localized in peroxisome, is induced in the cells grown on non-fermentable carbon sources, i.e. *n*-alkanes, propionate or acetate, than in the glucose-grown cells [24, 25]. The gene coding for ICL was

cloned [25] and, when introduced into another yeast *Saccharomyces cerevisiae*, 5'-upstream region of the gene (*UPR-ICL*) could function and regulate gene expression in response to carbon source [26, 27]. Deletion analysis of *UPR-ICL* identified two upstream activation sequences (UAS; region A1 and region A2) which can independently activate gene expression in the acetate-grown cells [28]. Region A2 contained a consensus sequence of carbon source response element (CSRE) which is an upstream activation sequence (UAS) identified in *ICL1* of *Saccharomyces cerevisiae* [29]. Expression of gene mediated by region A2 was impaired in the Δ*snf1* mutant of *Saccharomyces cerevisiae* [28], as is the case with CSRE-mediated gene expression [29]. These results led the author to interest in the relationship between induction of peroxisomal enzymes including ICL and function of *SNF1*. Moreover, relation between *SNF1* and peroxisome proliferation is also interesting, because Simon *et al*. reported that the *snf1* deletion mutant of *Saccharomyces cerevisiae* grown on oleic acid had no detectable peroxisomes [30]

In yeast, three *SNF1* homologs are presently identified. *SNF1* of *Candida glabrata* (*CgSNF1*) is necessary for the cell to utilize trehalose [31]. *SNF1* of *Kluyveromyces lactis* (*KlSNF1*) is necessary for the growth on non-fermentable carbon sources and some fermentable sugars (such as galactose or lactose) [32, 33]. Like the case of *ScSNF1*, *CgSNF1* and *KlSNF1* are dispensable for the glucose-grown cells. Different from other *SNF1*s, *SNF1* of *Candida albicans* (*CaSNF1*) is necessary even for the growth on glucose [34].

In this chapter, to elucidate the role of *SNF1* on the induction of enzymes localized in peroxisome or on the cell viability, *SNF1* of *Candida tropicalis* (*CtSNF1*) was cloned and characterized. Disruption of one allele of *CtSNF1* caused no significant change in the growth on glucose or sucrose. On the other hand, the growth and activity of some peroxisomal enzymes on *n*-alkane were slightly affected. Moreover, by a conditional expression of *CtSNF1*, it was shown that *CtSNF1* is an essential gene.

MATERIALS AND METHOD

Strains

Yeasts strains used in this study are listed in Table1. *Candida tropicalis* SU-2 (ATCC 20913) (*ura3/ura3*) [35, 36], derived from *Candida tropicalis* pK233 (ATCC 20336), was used as a parent strain for transformation. *Saccharomyces cerevisiae* strain MT8-1 (*MATa*,

ade, his3, leu2, trp1, ura3) [37] and its derivative strain AT002 (*MATa, ade, his3, leu2, trp1, ura3, snf1Δ::LEU2*) [28] were also used. *Escherichia coli* strain DH5α [38] was used for gene manipulation.

Media and growth conditions

Candida tropicalis was cultivated aerobically at 30°C in a medium containing glucose (16.5 g/L), galactose (16.5 g/L), sucrose (16.5 g/L), sodium acetate (13.6 g/L) or *n*-alkane mixture (C_{10} to C_{13}) (10 ml/L) as a sole carbon source [39, 40]. pH of respective medium was adjusted to 5.2 for glucose, galactose, sucrose, and *n*-alkane media or to 6.0 for acetate medium. Tween 80 (0.5 ml/L) was added in the *n*-alkane medium. The basic composition of medium was as follows: 5.0 g of $NH_4H_2PO_4$, 2.5 g of KH_2PO_4, 1.0 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $FeCl_3 \cdot 6H_2O$, and 1.0 ml of corn steep liquor per liter of tap water [39]. Cell growth was monitored by measuring light scattering at 570 nm.

Cloning of *SNF1* of *Candida tropicalis*

Two degenerative oligonucleotide primers compatible with the conserved regions of yeast *SNF1* genes [5'-GGTAAGTTGTACGC(C/T)GGTCCAGAAGT(C/T)GA(C/T)-G-T(C/T)TTG-3' and 5'-TCCAATGG(G/A)TA(G/A)GATCT(G/A)GATCT(G/A)ATAC-C(G/A)AAGTGCC-3'] were used for the amplification of a DNA fragment containing *CtSNF1* by PCR. The amplified DNA fragment of 833-bp was introduced into *Hinc*II site of pUC19 (named as pUC19S), where the region near start codon of *CtSNF1* is located on the *Eco*RI side. A biotinylated probe prepared from pUC19S by restriction enzyme digestion was used for screening of *CtSNF1* from λ-EMBL3 genomic DNA library of *Candida tropicalis* pK233, and subsequently eight probe-hybridizing clones were isolated. From one clone, a probe-hybridizing *Xba*I-*Pst*I fragment (3.3-kbp) was cut and subcloned into pUC19, and the resulting plasmid (pUC19-XP) was used to determine the nucleotide sequence of *CtSNF1*.

Construction of plasmids

1) pCtSNF1. The *Xba*I-*Pst*I fragment (3.3-kbp) of the genomic DNA fragment containing *CtSNF1* was introduced into the *Xba*I-*Pst*I sites of a plasmid pRS414 [41]. The resulting plasmid, named as pCtSNF1, was introduced into the *Saccharomyces cerevisiae* Δ*snf1* strain (AT002).

2) pKO1::ZUZ. To amplify a DNA fragment containing 409-bp of *CtSNF1* (region 664 to 1072; where A of the start codon was set as 1), PCR was performed using a primer, 5'-

Table 1. Strains used in this chapter.

Genotype			Reference number
Candida tropicalis			
pK233	*URA3/URA3*	*SNF1/SNF1*	22
SU-2	*ura3/ura3*	*SNF1/SNF1*	35, 36
KO-1(ZUZ)	*ura3/ura3*	*SNF1/snf1Δ::ZUZ*	This study
KO-1	*ura3/ura3*	*SNF1/snf1Δ::lacZ*	This study
KO-1G(ZUZ)	*ura3/ura3*	*snf1Δ::ZUZ-pGAL10-SNF1/snf1Δ::lacZ*	This study
KO-1G	*ura3/ura3*	*snf1Δ::lacZ-pGAL10-SNF1/snf1Δ::lacZ*	This study
Saccharomyces cerevisiae			
MT8-1	*MATa, ade, his3, leu2, trp1, ura3*		37
AT002	*MATa, ade, his3, leu2, trp1, ura3, snf1Δ::LEU2*		28

CAGCGAATTCTCTGTTGGCTTTTTCAATCACGG-3' and the M13 primer. pUC19S was used as a template DNA. The amplified fragment was cut by *Eco*RI and introduced into the *Eco*RI site of pZUZ [35]. To amplify a DNA fragment containing 425-bp *CtSNF1* (region 1072 to 1496), PCR was performed using a primer, 5'-GCTGGTCGACCAACTCCAACAAA-CCAATCTAAATC-3' and the M13 reverse primer. The amplified fragment was cut by *Sal*I and *Pst*I and introduced between the *Sal*I and the *Pst*I sites of pZUZ (pKO1::ZUZ, Fig. 3C). pKO1::ZUZ was cut by *Bam*HI and *Pst*I, and introduced into *Candida tropicalis* to disrupt *CtSNF1*.

3) pKO1G::ZUZ. Cloning of the *GAL1-10* promoter region from *Candida tropicalis* pK233 was performed as follows. Two degenerative oligonucleotide primers compatible with the conserved regions of *GAL1* gene [5'-GC(G/A)AC(G/A)ATCAA(G/A)GCACA(C/T)T-T(G/A)AAGT-A(G/A)TT(G/A)GCCCA-3'] and of *GAL10* gene [5'-TT(G/A)ATCAATT-C(G/A)AT(G/A)AC(G/A)GT(G/A)TG(G/A)GAACC(G/A)ATGTA-3'] were used for the amplification of a DNA fragment by PCR. A DNA fragment (1.3-kbp) was amplified, introduced into pUC19 (pUC19G) and the nucleotide sequence was determined (GenBank/EMBL/DDBJ database accession number AB019434). The amplified fragment had the ability of inducing the gene of its downstream when galactose was added to the medium.

To amplify a DNA fragment containing the *GAL10* promoter region, PCR was performed using following primers; 5'-TTCGGTCGACCTGATATGTGTGAGTGTGC-3' and 5'-ACATCTGCAGCTCTTGAGCTATACCAATCC-3'. pUC19G was used as a template. The amplified fragment was cut by *Sal*I and *Pst*I, and introduced into the *Sal*I and the *Pst*I sites of

pZUZ (pZUZG).

The promoter region of *CtSNF1* (region -300 to -1) was amplified using a primer, 5'-TCCGGAATTCTGACTGAACAATTCTAATGG-3' and the M13 primer. Amplification of a DNA fragment containing 969-bp *CtSNF1* (region 1 to 969) was performed using the following primers; 5'-TTCACTGCAGATGTCGGAGCAGAATCAAGG-3' and 5'-CAACGCATGCATAACGTCTTCGTCGATATCAATC-3'. In both cases, pUC19-XP was used as a template. The amplified fragment containing *CtSNF1* promoter was cut by *Eco*RI and introduced into the *Eco*RI site of pZUZG. The amplified fragment containing a part of *CtSNF1* was cut by *Pst*I and *Sph*I and introduced between the *Pst*I and *Sph*I sites of pZUZG, generating a plasmid pKO1G::ZUZ (Fig 3C). pKO1::ZUZ was cut by *Bam*HI and *Sph*I, and used to replace the promoter region of *CtSNF1*.

Enzyme and protein assays

Enzyme activities were determined as described in published papers; acyl-CoA oxidase [42], catalase and isocitrate lyase [43]. All cells were harvested at late exponential period. Protein concentration was assayed by Bradford method using bovine serum albumin as the standard.

Other methods

Transformation of *Candida tropicalis* was performed by the spheroplast method as reported by Kanayama *et al.* [35]. Southern blot analysis was carried out as described by Kurihara *et al.* [44]. General methods for gene manipulation and yeast genetics were used as described in general protocols [38, 45].

RESULT

Isolation of *CtSNF1*.

On the basis of amino acid sequences highly conserved among yeast *SNF1* homologs, two oligonucleotide primers were designed (see MATERIALS AND METHODS). Codon preference of *Candida tropicalis* was also considered in designing primers. Using genomic DNA of *Candida tropicalis* pK233 as template, a DNA fragment (833-bp) was amplified by PCR. This fragment contained a homologous sequence with other *SNF1*s. Using this DNA fragment as the probe, λ-EMBL3 genomic DNA library of *Candida tropicalis* pK233 was screened, and eight positive plaques were selected. From one clone, a probe-hybridizing

A

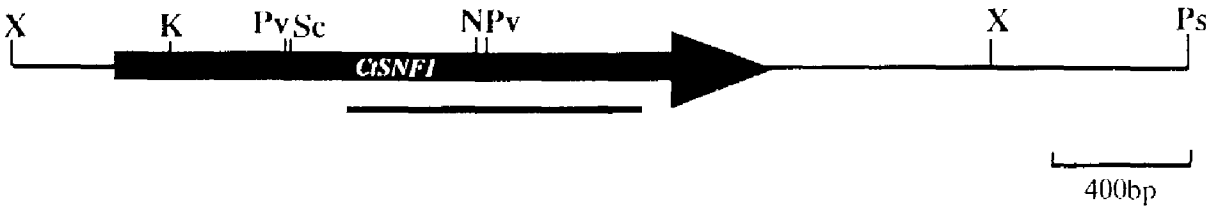


Fig.1 Restriction map of *CtSNF1* (A), and alignment of putative CtSNF1p amino acid sequence with its yeast homologs (B). In A, restriction sites: K, *Kpn*I; N, *Nco*I; Ps, *Pst*I; Pv, *Pvu* II; Sc, *Sca*I; X, *Xba*I. Black bar indicates the probe region (833-bp). In B, asterisks indicate the amino acid residues identical in all proteins. Dots indicate the amino acid residues with higher conservation in all proteins. Region of activation segment is *boxed*. ADF/APE motif and the conserved threonine residue in activation segment are written in *bold*. Regions of Snf4p- and Gal83p/Sip1p/Sip2p-binding sites for ScSnf1 are *underlined*.

*Xba*I-*Pst*I fragment (3.3-kbp, Fig. 1A) was cut and subcloned into pUC19. Sequence analysis revealed the existance of one open reading frame (*CtSNF1*) of 1,857-bp encoding a putative 619-amino acid protein (CtSnf1p, Fig. 1B). CtSnf1p showed a notably high similarity (86.0% identity) to Snf1p of *Candida albicans* (CaSnf1p). On the other hand, identity with *Saccharomyces cerevisiae* Snf1p (ScSnf1p) was 61.2%. At its amino terminus of CtSnf1p, there was a stretch of histidine residues conserved in most of the yeast Snf1p. Alignment of CtSnf1p with other yeast Snf1p revealed that kinase domain which occupies amino-terminal half are strikingly conserved among yeast Snf1p homologs (Fig. 1B). As for ScSnf1p, the Snf4p- and Sip1p/Sip2p/Gal83p-binding sites were determined between amino acids residues 392-518 [46] and 515-633 [21] of ScSnf1p, respectively, by two-hybrid assay. The carboxy-terminal region including Sip1p/Sip2p/Gal83p-binding site of ScSnf1p is highly conserved among yeast Snf1p, whereas the region including Snf4p-binding site is not (Fig. 1B).

Complementation of a Δ*snf1* strain of *Saccharomyces cerevisiae* by *CtSNF1*.

A *Xba*I-*Pst*I fragment (3.3-kbp) containing *CtSNF1* coding region, the 5'-flanking region, and the 3'-flanking region was subcloned into pRS414 [41] and the resulting plasmid

B

Fig.1. (Continued).

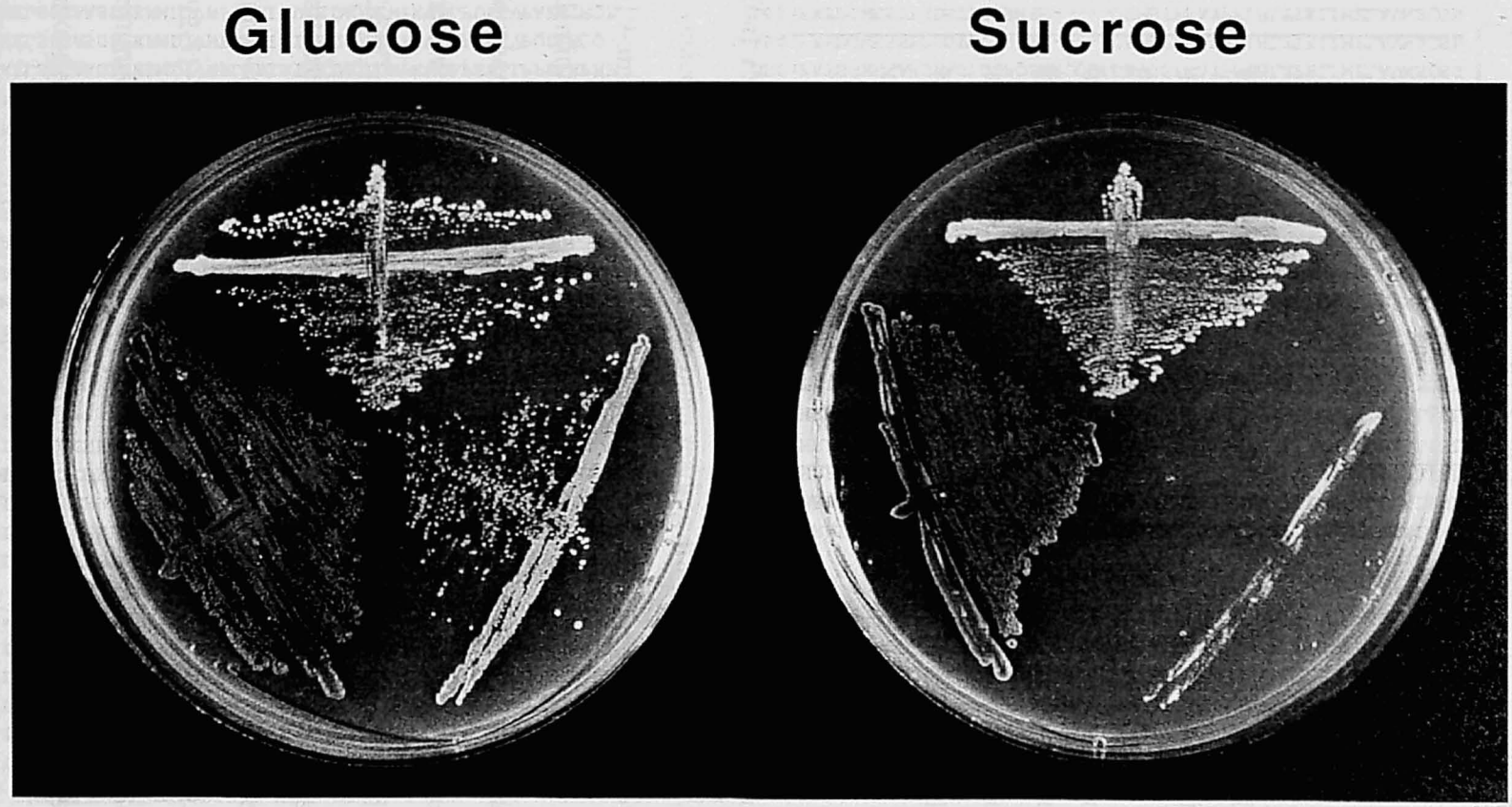
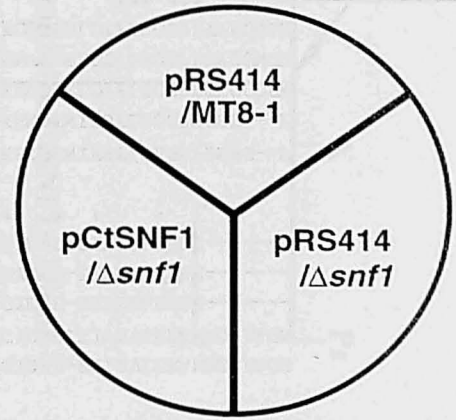


Fig.2 Complementation of a *Saccharomyces cerevisiae snf1* deletion mutant with the *CtSNF1*. *Saccharomyces cerevisiae* wild-type strain (MT8-1) and AT002 (Δ*snf1*) were used. Cells were grown at 30 °C on either selective medium (0.67 % Yeast nitrogen base without amino acids (Difco) with 0.002% adenine sulfate, 0.002% uracil, 0.002% L-histidine-HCl, and 0.003% L-leucine) containing 2% of glucose (*left*) or sucrose (*right*).



(named as pCtSNF1) was introduced into the Δ*snf1* strain of *Saccharomyces cerevisiae* (AT002, Table 1). Transformants were selected for tryptophan prototorophy and the ability of sucrose utilization was tested using a plate containing sucrose as a sole carbon source. The AT002 strain transformed with pRS414 did not grow on the plate containing sucrose. On the other hand, the Δ*snf1* strain transformed with pCtSNF1 recovered the ability of sucrose utilization (Fig. 2). Furthermore, an increase of isocitrate lyase activity, which is impaired in the *snf1* strain grown on non-fermentable carbon sources, was restored for the Δ*snf1* strain transformed with pCtSNF1 but not for that transformed with pRS414, when grown on acetate. These results indicate that, like the other yeast Snf1p, CtSnf1p is a functional homolog.

Construction of a *SNF1/snf1* heterozygote strain (KO-1) of *Candida tropicalis*.

To investigate the cellular function of CtSnf1p, gene disruption of *CtSNF1* was performed. General technique to disrupt a particular gene(s) of *Candida tropicalis* was employed [35]. *Candida tropicalis* SU-2, which is a *ura3*⁻ derivative of *Candida tropicalis* pK233 [35, 36], was used as the parent strain. A plasmid, pKO1::ZUZ (see MATERIALS AND METHODS and Fig. 3C) which is designed to disrupt *CtSNF1* (Fig. 3A), was constructed, and introduced into *Candida tropicalis* SU-2. Several Ura⁺ colonies were selected and correct integration of the disruption vector was confirmed by Southern blot analysis. One clone [KO-1(ZUZ)] was selected, and inoculated on a minimal medium containing 5-fluoroorotic acid (5FOA, 0.75 g/L) for 4 days in order to pop out *URA3*. The isolated 5FOA-resistant colonies were subjected to Southern blot analysis to confirm correct genetic rearrangement around *CtSNF1* (Fig. 4). One clone (named as KO-1) was selected, in which one *CtSNF1* allele was disrupted.

Using pKO1::ZUZ, disruption of the second *CtSNF1* allele was performed for KO-1. However, all Ura⁺ colonies were shown to contain intact *CtSNF1* allele as checked by Southern blot analysis. *CaSNF1*, recently reported, is an essential gene even for the cells grown on glucose [34]. This feature differs from other yeast *SNF1*s which are dispensable in the glucose-grown cells. Taking a high similarity of *CtSNF1* with *CaSNF1* in amino acid sequence into account, *CtSNF1* is also likely to be an essential gene.

Because Snf1p is necessary for the growth on diverse carbon sources, the growth of KO-1 on several carbon sources was compared with the parent strain. In respect to *Candida albicans*, the *SNF1/snf1* heterozygote strain showed reduced growth on glucose or sucrose [34]. When KO-1 cells were grown on glucose, sucrose or acetate, no significant difference

A

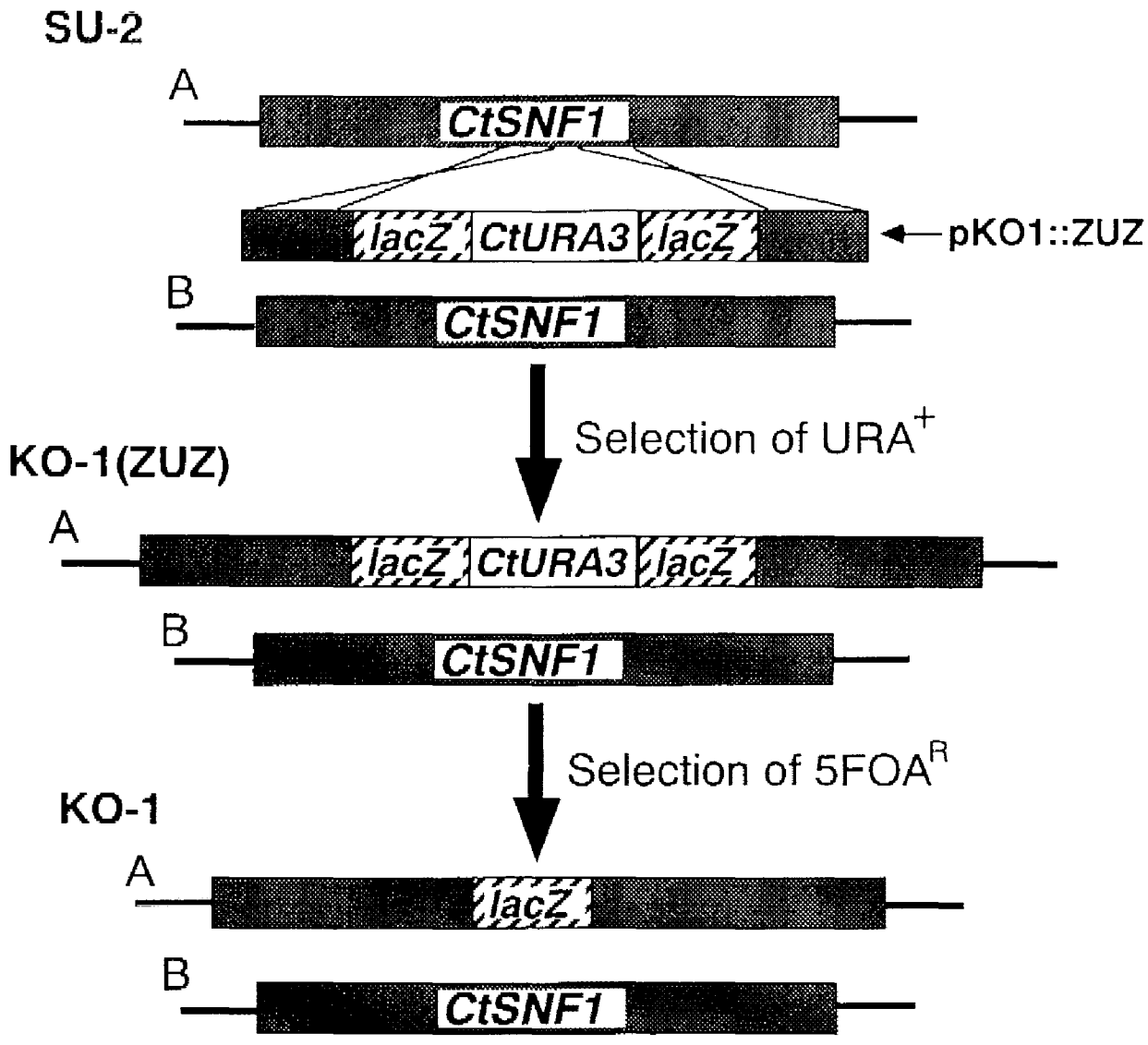


Fig.3. (A) Schematic representation of the construction of a strain in which one allele of *CtSNF1* is disrupted (KO-1). Genomic structures around *CtSNF1* are described for each strain constructed. For the discrimination, each allele is named as A and B. Name of the respective strain is indicated at left. **(B) Schematic representation of the construction of a strain in which promoter region of *CtSNF1* is replaced with *GAL10* promoter (KO-1G).** Descriptions are same as in (A). **(C) Partial restriction map of *CtSNF1* and the plasmids used in this study.** The homologous regions in plasmids with *CtSNF1* were indicated by arrows. *pCtSNF1*; the promoter region of *CtSNF1*. *pGAL10*; the promoter region of *GAL10* of *Candida tropicalis*.

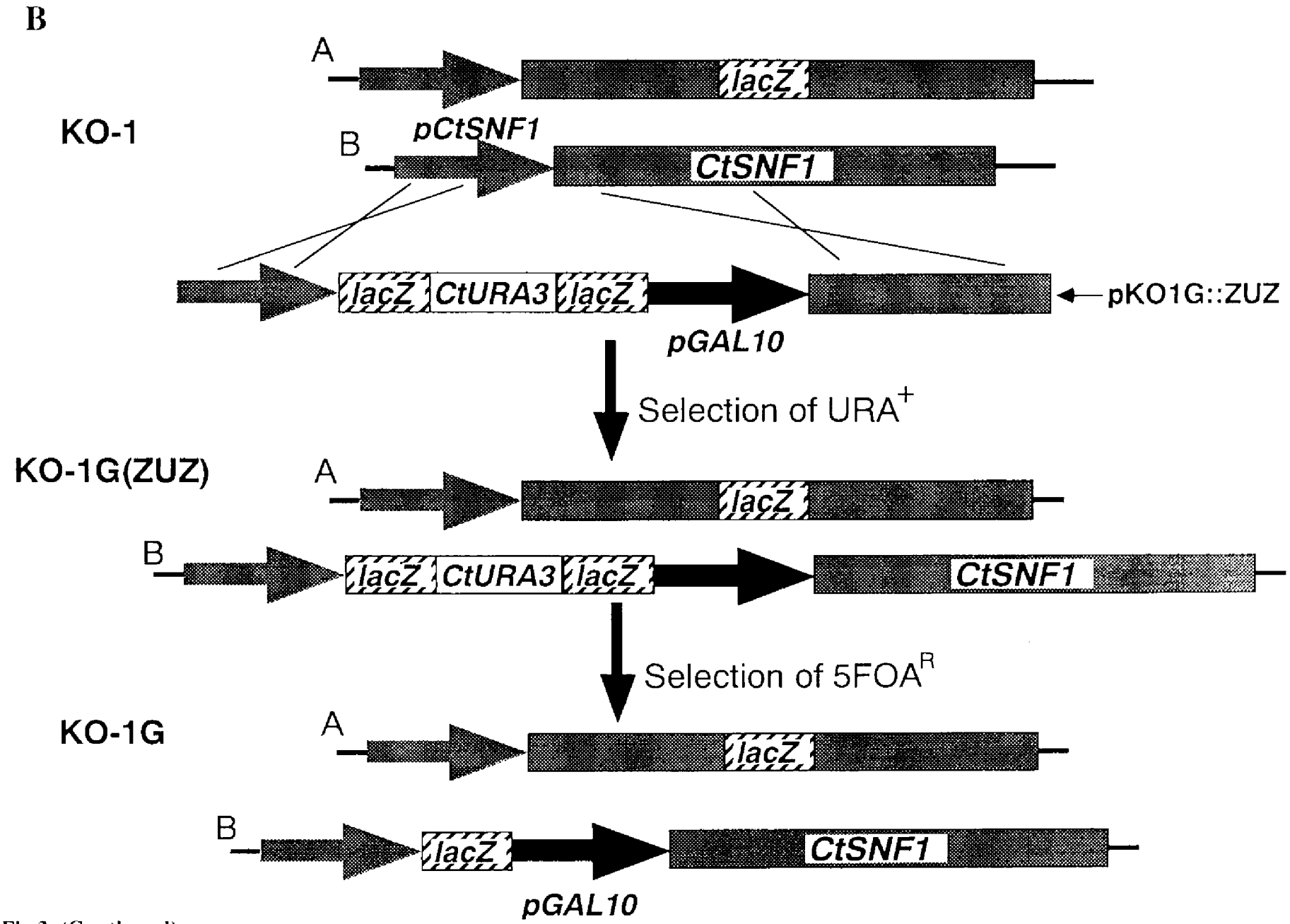


Fig.3. (Continued).

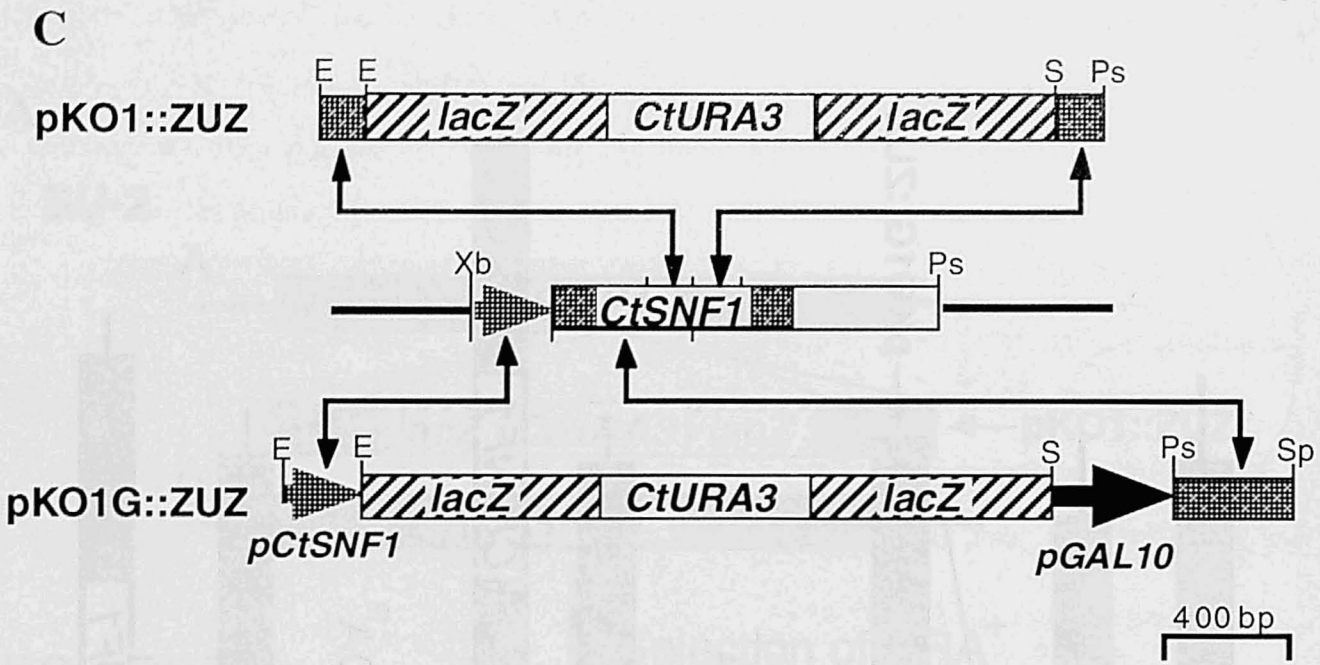


Fig.3. (Continued).

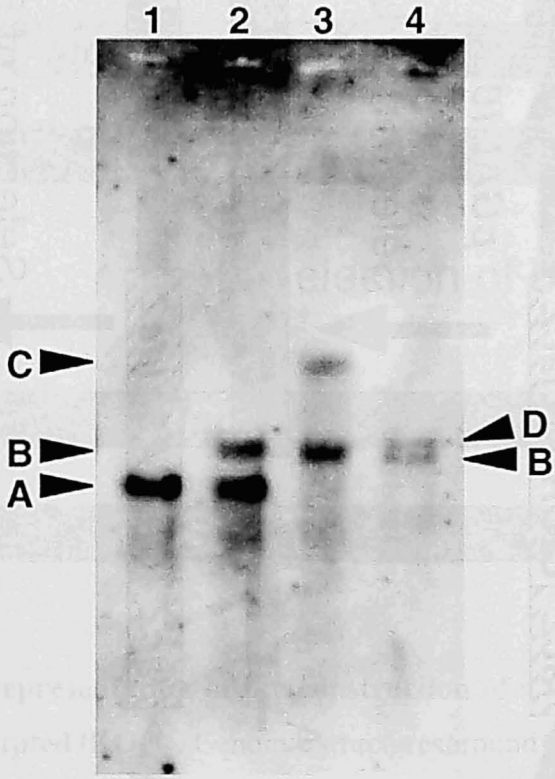


Fig.4 Southern blot hybridization of *Sph*I digested DNA with *CtSNF1* probe. *Lane 1*, SU-2; *lane 2*, KO-1; *lane 3*, KO-1G(ZUZ); *lane 4*, KO-1G. Band A; original DNA fragment containing *CtSNF1* (7,000-bp). Band B; DNA fragment containing *CtSNF1* with *lacZ* insertion (8,900-bp). Band C; DNA fragment containing *CtSNF1* inserted with *GAL10* promoter as well as *lacZ-URA3-lacZ* cassette (13,300-bp). Band D; DNA fragment containing *CtSNF1* inserted with *GAL10* promoter and *lacZ*. (9,730-bp).

Table 2. Effect of deleting one allele of *CtSNF1* on peroxisomal enzyme activities. [a]ACO, acyl-CoA oxidase; [b]ICL, isocitrate lyase.

1. *n*-Alkane

Strain	Catalase (mmol min^{-1} mg^{-1})	ACO[a] (nmol min^{-1} mg^{-1})	ICL[b] (nmol min^{-1} mg^{-1})
SU-2	2570	1150	132
KO-1	2060	785	128

2. Acetate

Strain	Catalase (mmol min^{-1} mg^{-1})	ACO[a] (nmol min^{-1} mg^{-1})	ICL[b] (nmol min^{-1} mg^{-1})
SU-2	532	73.6	250
KO-1	250	52.4	249

was observed (Fig. 5A). On the other hand, the *n*-alkane-grown KO-1 showed reduced cell growth compared with the parent cells. The activities of three peroxisomal enzymes were then compared in the acetate- and *n*-alkane-grown cells. The activities of catalase and acyl-CoA oxidase, both of which are enzymes of the β-oxidation cycle, are induced in the *n*-alkane-grown cells [23], whereas isocitrate lyase, which is a key enzyme of the glyoxylate cycle, is induced in the acetate-grown cells [24]. Catalase and acyl-CoA oxidase activities were decreased 47 to 80 % in KO-1 cells against the parent cells. On the other hand, the activity of isocitrate lyase was almost comparable to the parent cells (Table 2).

Construction of a strain in which the promoter region of *CtSNF1* was replaced with *GAL10* promoter (KO-1G) and its characterization.

In order to study the effect of total deletion of *CtSNF1*, a strain in which *CtSNF1* can be conditionally expressed was constructed. KO-1G is a strain in which the promoter region of *CtSNF1* was replaced with *GAL10* promoter, and was constructed from KO-1 using a plasmid pKO1G::ZUZ followed by the *URA3* popping out (Fig. 3B, 3C). The correct construction was confirmed by Southern blot analysis (Fig. 4). The *GAL10* promoter region

of *Candida tropicalis* was cloned by the method described in MATERIALS AND METHODS. *GAL10* promoter induces transcription on the addition of galactose, while by other carbon sources, such as glucose, glycerol, lactate or *n*-alkane, this induction does not occur.

The KO-1G strain successfully constructed was then compared for its growth on different carbon sources (glucose, galactose, *n*-alkane, and acetate) (Fig. 5B). Galactose-containing medium was used for precultivation. The galactose-grown KO-1G strain showed completely the same growth kinetics with the parent cells. However, as for the other carbon sources including glucose, stop of growth was observed for KO-1G strain around 14 h of cultivation. These results indicates that *CtSNF1* is an essential gene for growth even in the glucose-grown cells as well as in the *n*-alkane- and acetate-grown cells.

DISCUSSION

This chapter deals with the isolation of an Snf1 homolog from *Candida tropicalis* and its characterization. CtSnf1p showed a sequence similarity with other Snf1p from yeasts, and notably high similarity in amino acid sequence (86.0% identity) with the Snf1p of *Candida albicans* (CaSnf1p) was observed. Moreover, like other Snf1 homologs, CtSnf1p had an ability to complement Δ*snf1* mutant of *Saccharomyces cerevisiae*. These results suggest the existence of a similar mechanism concerning Snf1 protein kinase cascade between these yeasts. Snf1p of *Saccharomyces cerevisiae* (ScSnf1p) is activated in the absence of glucose, while it is inactivated in the presence of glucose [47, 48]. This is consistent with the fact that *ScSNF1* is dispensable in the glucose-grown cells. On the other hand, *CtSNF1* is essential for the cell viability like the case of *CaSNF1* [34]. Therefore, it is of interest to know whether or not the activity of CtSnf1p is regulated by the presence of glucose. Many protein kinases are activated by the phosphorylation on a residue(s) located in a particular segment termed "activation segment" [49]. The activation segment is defined as the region spanning conserved DFG and APE/SPE motifs, and inside of the activation segment, there is a conserved threonine residue [49]. The activation segment of CtSnf1p locates from residues 192 to 218 (Fig. 1B), and this region is highly conserved not only among the yeast Snf1p but with the AMP-activated protein kinase (AMPK), a mammalian homolog of Snf1p [50]. As for AMPK, a threonine residue (T^{172}), which lies inside of the activation segment, is phosphorylated by the action of AMP-activated protein kinase kinase (AMPKK) [51], indicating possible existence of a

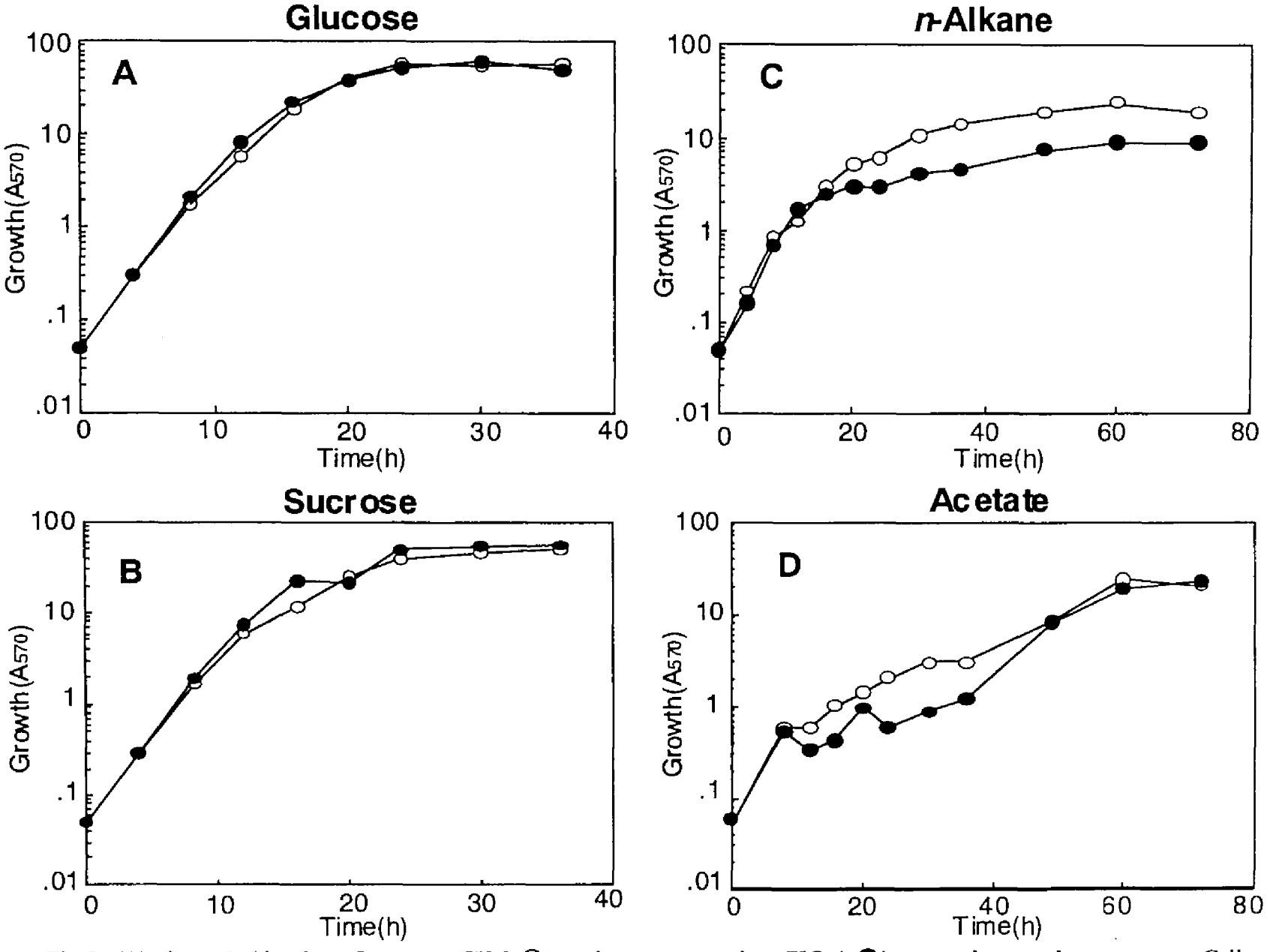


Fig.5. (A) Growth kinetics of parent (SU-2;○) and mutant strain (KO-1;●) on various carbon sources. Cells were precultivated by glucose.

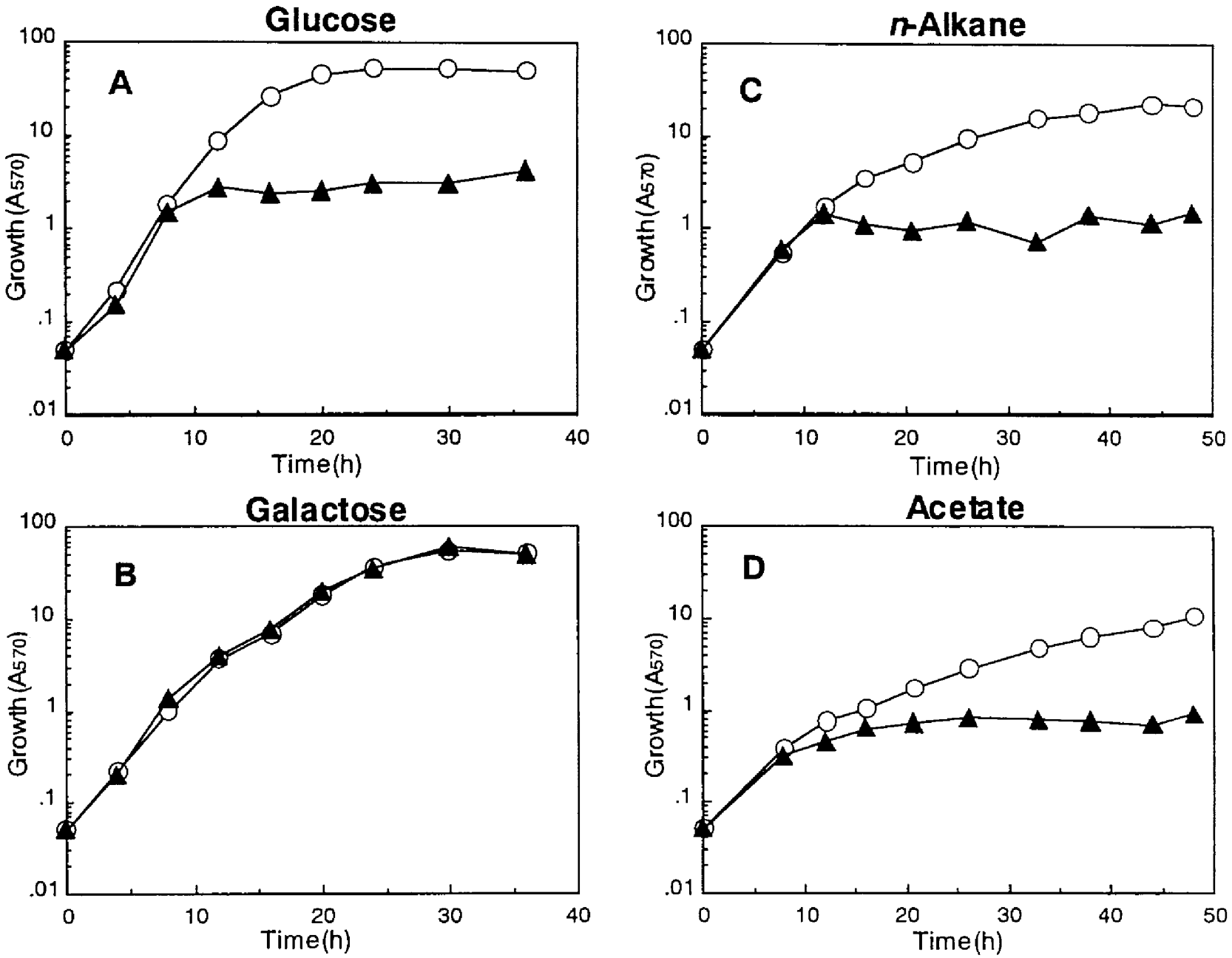


Fig.5. (B) Growth kinetics of parent (SU-2;○) and mutant strain (KO-1G;▲) on various carbon sources. Cells were precultivated by galactose.

upstream kinase cascade through the activation segment. Also for *Saccharomyces cerevisiae*, the existence of Snf1 kinase kinase is genetically suggested [52]. Judging from structural similarity, there is a possibility that the kinase activity of CtSnf1p is regulated by glucose through phosphorylation of the activation segment by upstream unidentified kinase.

Deletion of one allele of *CtSNF1* (strain KO-1) did not cause any growth retardation on the media containing glucose or sucrose, which is different from the case of *CaSNF1* where 39 % of growth retardation was observed for the *SNF1/snf1* heterozygote on these carbon sources [34]. On the other hand, KO-1 cells grown on *n*-alkane showed a slight growth retardation comparing with the parent cells, and the enzymatic activities of acyl-CoA oxidase and catalase, both of which are enzymes of the β-oxidation cycle, were also lower while that of isocitrate lyase did not changed. The same type of change in enzyme activity was also observed for the acetate-grown KO-1 cells. In *Saccharomyces cerevisiae*, derepression of isocitrate lyase (encoded by *ICL1*) is under the control of ScSnf1p [53], while induction of catalase (encoded by *CTA1*) and acyl-CoA oxidase (encoded by *FOX1*) on oleic acid are not dependent on ScSnf1p [30, 54]. Although the different effect of deleting one *CtSNF1* allele on each enzyme activity is not clear at present, the function of CtSNF1p seems to be similar but not completely the same as ScSnf1p or CaSnf1p. As *CtSNF1* is an essential gene for growth, the employment of a conditional system, such as exchanging of *CtSNF1* promoter region, the employment of a temperature-sensitive *CtSNF1* allele or antisence RNA for *CtSNF1*, would be effective to elucidate the function of *CtSNF1* on the induction of these enzymes.

SUMMARY

SNF1 of *Saccharomyces cerevisiae* is an essential gene for the derepression of glucose repression. Homolog of *SNF1* was isolated from *n*-alkane-assimilating and asporogenic diploid yeast, *Candida tropicalis* (*CtSNF1*). *CtSNF1* could complement the Δ*snf1* mutant of *Saccharomyces cerevisiae*. The method for introducing the exogenous DNA into *Candida tropicalis* [*Kanayama et al. J. Bacteriol. vol.180 p.690 (1998)*] was employed to construct *SNF1/snf1* heterozygote and *snf1/snf1* homozygote strains. The *SNF1/snf1* heterozygote successfully constructed was named as KO-1. On the other hand, disruption of second *CtSNF1* allele was unsuccessful, suggesting a possibility that *CtSNF1* is an essential gene for *Candida*

tropicalis. Therefore, in order to control the expression of *CtSNF1*, a strain in which promoter region of *CtSNF1* was replaced with *GAL10* promoter of *Candida tropicalis* was constructed (named as KO-1G), and the properties of KO-1 and KO-1G strains were compared with the parent strain. In KO-1 strain, no significant difference could be observed for the growth on glucose, sucrose or acetate, but growth retardation occurred on *n*-alkane, which is a carbon source inducing peroxisome. The activities of catalase and acyl-CoA oxidase in the cell lysate were decreased 47 to 80 % against the parent strain, while the activity of isocitrate lyase was almost comparable. As for KO-1G strain, while normal growth was observed on galactose, transfer of cells into glucose, acetate or *n*-alkane medium lead the strain to stop growing. From these results, *CtSNF1* is an essential gene for cell viability on any carbon source.

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GENERAL CONCLUSION

The present study has been carried out to elucidate the regulation mechanism of the isocitrate lyase gene promoter of an *n*-alkane-assimilating yeast, *Candida tropicalis* (*UPR-ICL*) and its application to heterologous gene expression.

The gene coding for β-galactosidase from *Escherichia coli* (*LacZ*) was expressed in *Saccharomyces cerevisiae* using *UPR-ICL*. Expression of *LacZ* was repressed by glucose and enhanced over 300-fold by acetate. The expression level of β-galactosidase reached 6.5 % of the total soluble protein and was comparable to that with *GAL1* as a promoter. With *UPR-ICL*, the smaller isoform of rat glutamate decarboxylase (GAD65) was highly produced in *Saccharomyces cerevisiae* as a soluble and active form. A multicopy vector, pWI3, was constructed which contains multicloning site between *UPR-ICL* and the transcriptional terminator of the isocitrate lyase gene (*TERM-ICL*), as well as autonomous replication sequence and maker gene which are both functional in *Saccharomyces cerevisiae*.

Human histidine decarboxylase was expressed in *Saccharomyces cerevisiae* using pWI3. Specific activity in the crude cell lysate reached 210 (pmol min^{-1} mg^{-1}), and this value was twice and three times higher than that in the crude extract of mast cells and in mastocytoma P-815 culture cells, respectively.

A truncated fragment of the cycloinulo-oligosaccharide fructanotransferase (CFTase) gene of *Bacillus circulans* MCI-2554 was expressed in *Saccharomyces cerevisiae* under the control of *UPR-ICL*. Secreted recombinant CFTase protein (*Sc*CFTase) was purified. *Sc*CFTase2 which is the major product of the expression system, was N-glycosylated and exhibited a significant increase in thermostability comparing with CFTase purified from *Bacillus circulans* MCI-2554; over 50% of *Sc*CFTase 2 activity was retained even after 30 min of incubation at 80 °C. A strain of *Saccharomyces cerevisiae* was constructed which had two copies of CFTase gene integrated into its chromosomes (CF/HW2A), and production of *Sc*CFTase by the CF/HW2A strain reached 391 U per liter of culture at 120 h, which

corresponded to 8.40 mg of protein per liter, by shake-flask cultivation.

To search for factors regulating *UPR-ICL*-mediated transcription in *Saccharomyces cerevisiae*, a mutant was isolated which was unable to derepress *UPR-ICL* in acetate medium. The gene that complemented this mutation (*FIL1*) encoded a 230-amino acid protein (Fil1p) showing similarity to ribosome recycling factors (RRFs) of prokaryotes with mitochondrial-targeting sequence at its N-terminus. Fil1p was suggested as a protein necessary for mitochondrial protein synthesis through its possible function as a mitochondrial RRF. Furthermore, the necessity of normal mitochondrial respiratory chain was found for the derepression of the genes encoding the key enzymes of the glyoxylate cycle and gluconeogenic pathway, which indicates the presence of an inter-organelle communication pathway between mitochondria and the nucleus.

To search for regulation factors for the enzymes controlled by glucose repression (including isocitrate lyase) in *Candida tropicalis*, a homolog of *SNF1* (*CtSNF1*) was isolated. The *SNF1/snf1* heterozygote (KO-1) was constructed, and the strain showed the retardation of growth on *n*-alkane and the decreased activities of catalase and acyl-CoA oxidase in the cell lysate against the parent strain, while the activity of isocitrate lyase was almost comparable. Disruption of the second *CtSNF1* allele was unsuccessful. Therefore, a strain in which promoter region of *CtSNF1* was replaced with *GAL10* promoter was constructed (named as KO-1G), in order to control the expression of *CtSNF1*. Growth of KO-1G can be observed only for galactose, indicating that *CtSNF1* is an essential gene for cell viability.

PUBLICATION LIST

Part I

Chapter 1

A novel heterologous gene expression system in *Saccharomyces cerevisiae* using the isocitrate lyase gene promoter from *Candida tropicalis*.

Kanai, T., Atomi, H., Umemura, K., Ueno, H., Teranishi, Y., Ueda, M. & Tanaka, A.
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Chapter 3

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Part II

Chapter 1

A regulatory factor, Fil1p, involved in glucose repression/derepression in *Saccharomyces cerevisiae*: a possible mitochondrial protein necessary for protein synthesis in mitochondria.

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Chapter 2

Genetic evaluation of the function of *SNF1* in *Candida tropicalis*

Kanai, T., Ogawa, K., Ueda, M. & Tanaka, A.
in preparation

(OTHER PUBLICATIONS)

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